In vivo Implantation
Murine Modelling

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Attestation of Authorship

"I hereby declare that this submission is my own work and that to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been accepted for the award of any other degree or diploma of a university or other institution of higher learning."

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Auckland

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Abstract

Ethical and legal restrictions limit human implantation knowledge. Alternative tools, such as retrospective clinical studies, *in vitro* three-dimensional cell-culture modelling, and *in vivo* implantation animal modelling provide some insight. While *in vivo* implantation murine models have proven useful, none are recognised as standard. Therefore, the overall research objective was to gain an understanding of improved standardization of *in vivo* implantation murine models, suitable to explore implantation and preliminary studies surrounding *In Vitro* Fertilization (IVF) intervention potential.

A selection of models, termed optimal or suboptimal by their level of implantation, were examined and compared to natural mouse pregnancy. Embryo transfer (ET) outcomes on day-17 post-coitus (pc) and the ability of the most suitable optimal and suboptimal models to detect consequences of manipulated embryos, an IVF-intervention, were explored. Significant inconsistencies within suboptimal models led to investigating the beginning of endometrial receptivity within natural pregnancy, and synchronous and asynchronous uterine ET. Assessed time-points were set to capture the pre-receptive and receptive endometrial phases. Examining at least three mice per time-point, achieving 80% statistical power, ensured statistical robustness.

This research extended current knowledge in three ways. Firstly, ETs performed at two-hourly intervals, demonstrated significant influence of the distinct moments of coitus, ET and autopsy on implantation rate consistency. Previous research, which specified the day of transfer rather than hours pc, was unable to reveal such influence. Finer control of coitus, ET and autopsy demonstrated improved implantation rate consistency, necessary for model standardization. Implementation within wider research could improve statistical robustness and facilitate greater comparison between research groups.

Secondly, assessed time-points extended beyond previous the literature, which did not report endometrial receptivity within natural pregnancy prior to 92 hours pc. This study reported endometrial receptivity by 88 hours pc. No mean zero baselines were also observed, suggesting endometrial receptivity could begin prior to 88 hours pc. A four hour difference is significant, given murine endometrial receptivity is considered at least 12, but less than 24 hours long, translating to a potential 33% increase in known implantation opportunity. Data collection surrounding the beginning of endometrial receptivity within natural pregnancy, and synchronous and asynchronous uterine ET, revealed reasons for implantation rate inconsistencies, not previously observed in single-point data sets; contributing towards the first point.

Thirdly, this is the first report of early endometrial receptivity, following the transfer of mature pre-implantation embryos directly into uteri, whose endometrium would not normally be exposed to embryos at that time. Only one other study, which performed oviductal transfers, demonstrated similar phenomena. Due to the lack of uterine ET evidence, investigators reasoned oviductal transport of pre-implantation embryos was responsible. However, uterine ET bypasses oviductal transport. Therefore, early endometrial receptivity was likely due to the premature uterine presence of mature pre-implantation embryos and not dependent on their oviductal transport.

This research provided robust insight into improved *in vivo* implantation murine model standardization. Model consistency could be improved by strictly controlling the time of coitus, ET and autopsy. Research suggested endometrial receptivity began prior to 88 hours pc. Findings provided further evidence that pre-implantation embryos can influence the timing of endometrial receptivity. Exploiting findings could provide opportunities to expand implantation knowledge and explore commercial benefits of IVF-interventions with more efficiency and reliability.

Abbreviations

ART	Assisted reproductive technology
CL	Corpus luteum
E	Estrogen
ET	Embryo transfer
EthOH	Ethanol
Ex	Embryo developmental age x-number of days pc
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
HA	Hyaluronan
hCG	Human chorionic gonadotrophin
HMW-HA	High molecular weight hyaluronan
ICM	Inner cell mass
IP	Intraperitoneal
IU	International units
IVF	*In vitro* fertilization
LH	Luteinizing hormone
N	Naturally ovulated
P	Progesterone
pb	Post-birth
pc	Post-coitus
PDZ	Primary decidual zone
PMSG	Pregnant mare serum gonadotrophin
Prepub	Human chorionic gonadotrophin, Chorulon, 1500 IU freeze dried powder
Rx	Recipient mouse endometrium developmental age x-number of days pc
SDZ	Secondary decidual zone
SO	Superovulated / superovulation
TD	Trophectoderm
U	Urinary
ZP	Zona pellucida
2D	Two-dimensional
3D	Three-dimensional

1   Introduction

1.1 Rationale

Very few events in life are more pivotal and fascinating than pregnancy and the birth of a newborn baby. However, it seems ironic that with a world population of over seven billion people, so little knowledge surrounds embryo implantation, a crucial step towards successful pregnancy. The ethical and legal constraints surrounding human research lead to this limitation (Lee & DeMayo, 2004; Rogers, 1995). For this reason, scientists rely on alternative tools to elucidate what might occur in human implantation. Some of which include: 1) data associated with clinical interventions; 2) *in vitro* modelling of trophectoderm-endometrial epithelial interaction; and 3) *in vivo* implantation animal modelling (Bentin-Ley & Lopata, 2000; Carson, et al., 2000; Makker & Singh, 2006). Although each tool provides substantial insight into human implantation, only *in vivo* implantation animal modelling can illuminate an *in utero* perspective.

A vast number of species enable *in vivo* exploration of implantation. Although none are truly analogous, as disclosed within Section 2.2.1, the unique attributes of each species facilitate extrapolation of distinct aspects of human implantation. Mice offer an attractive option, where their experimental output is high and expense low (Carson, et al., 2000). In addition, the experimental techniques association with murine exploration of implantation are well established (Hogan, Beddington, Costantini, & Lacy, 1994). Moreover, comprehensive knowledge surrounds the murine genomic profile, enabling in-depth exploration of the molecular aspects of implantation (Carson, et al., 2000; Lee, et al., 2007).

Despite the numerous *in vivo* implantation murine models available, none are recognized as standard. An extensive literature review, disseminated within Section 2.2, revealed four potential reasons for this lack of standardization. Potential reasons included: 1) a lack of reported statistical analysis suitable to determine mouse-to-mouse consistency within studies; 2) inconsistent implantation rates between research groups that utilised comparable models; 3) a lack of clarity surrounding the beginning of endometrial receptivity, critical for successful implantation; and 4) insufficient exploration surrounding the impact of altered embryo-endometrial developmental synchrony, in terms of hours not days, on the timing of endometrial

receptivity and resulting implantation rates. Model standardization would strengthen experimental data; enable greater comparison between research groups and aid development of effective research platforms, useful to explore implantation and preliminary studies surrounding IVF-intervention potential. For example: 1) investigating whether IVF-interventions were detrimental to ET outcomes; and 2) could that intervention restore suboptimal implantation rates to normal levels. Therefore, the overall research objective was to gain insight into improved standardization of *in vivo* implantation murine models that were suitable for the purposes specified above.

It is also important to note that within this research the term IVF refers to the entire cycle and not just the fertilization process. Interventions can focus on one aspect of IVF, for example embryo transfer media, or GnRH antagonist adminstration, which prevents early luetinzation. Yet, each technique is can be essential for a successful IVF cycle. Therefore, within this research, preliminary findings surrounding IVF-intervention potential, suggest its potential to improve IVF cycle success. Section 1.2 will highlight research objectives in greater detail.

1.2 Objectives

The overall purpose of this research was to gain an understanding into improved standardization of *in vivo* implantation murine models, which were suitable to explore implantation and preliminary studies surrounding IVF-intervention potential. This was achieved by the following:

1. Examination and comparison of a selection of *in vivo* murine implantation models with natural mouse pregnancy. Their levels of implantation classified models as optimal or suboptimal. Examination included ET outcomes on day-17 pc, and detailed assessment of the most suitable Optimal and Suboptimal model's ability to detect consequences of manipulated embryos, an IVF-intervention.
2. A methodical investigation of the beginning of endometrial receptivity within natural mouse pregnancy, and asynchronous and synchronous uterine ET.

1.3 Outline of thesis

The subsequent section is an introduction to the structure and content of this thesis. This includes an extensive literature review; two chapters that address the overall research objectives, which included a critical analysis and discussion of their results; concluding remarks and suggestions for future work.

Chapter 2 Literature review

An initial background to endometrial-embryo development and implantation is presented within Chapter 2. Such knowledge forms the foundation to the subsequent literature review, which explores optimal and suboptimal levels of murine implantation. While the literature revealed numerous *in vivo* implantation murine models, none were recognized as standard. This chapter concludes with a detailed account the factors contributing towards the lack of standardization.

Chapter 3 Materials and methodology

All manufacturer details for laboratory equipment and reagents have been provided in Chapter-3. Reagent preparation, mouse colony details and experimental procedures have also been provided.

Chapter 4 Results - Optimal and suboptimal in vivo implantation murine models

Standardization of *in vivo* implantation murine models, suitable to explore IVF-intervention potential, required both consistent implantation rates and an ability to detect consequences of that intervention. Gaining fundamental knowledge surrounding mean murine implantation rates was a necessary step. For this reason, the outcomes of natural mouse pregnancy on day-17 pc were compared with a number of optimal and suboptimal *in vivo* implantation murine models, which were classified by their level of implantation. Models were generated by altering their embryo-endometrial developmental synchrony and included: 1) ET into SO recipient mice (advanced endometrium); 2) progesterone antagonist administration following ET (delayed endometrium); and 3) synchronous and asynchronous ET (the purposeful shift of donor embryo and recipient mouse endometrial developmental age at the time of transfer). The information gathered provided foundational knowledge that indicated current model consistency.

Generating consistent models was only part of the standardization process. Before any model could be used to explore the potential of any IVF-intervention, it was important that the model was able to detect consequences of that intervention. This research chose to focus on embryo manipulation, a potential IVF-intervention. Therefore, the most suitable optimal and suboptimal *in vivo* implantation murine models were assessed for their ability to detect negative and positive consequences of manipulated embryos.

Chapter 5 Results – Refinement of *in vivo* implantation murine models

As work progressed, substantial mouse-to-mouse variability was observed within models that achieved low levels of implantation. In depth literature reviews revealed little regarding model consistency, but instead revealed gaps in knowledge. This included a lack of clarity surrounding the beginning of endometrial receptivity and how it's timing was influenced by altered embryo-endometrial developmental synchrony. Therefore, Chapter 5 methodically investigates the beginning of endometrial receptivity within natural mouse pregnancy, and synchronous and asynchronous uterine ET. The information gathered is then used to determine the requirements necessary for model standardization and gain further insight into fundamental knowledge surrounding endometrial receptivity.

Chapter 6 – Discussion

Critical analysis of Chapters 4 and 5 are presented in Chapter 6.

Chapter 7 – Concluding remarks

Chapter 7 draws together the findings of this research to form the concluding remarks. First the deficiencies of existing optimal and suboptimal *in vivo* implantation murine models are addressed. Second, a new understanding of *in vivo* implantation murine model standardization and fundamental knowledge surrounding endometrial receptivity is summarised.

Chapter 8 Suggestions for future research

Any potential research, which follows on from current findings, is addressed within Chapter 8.

2   Literature review

Prior to experimentation, it was first necessary to gain a clear foundation of normal endometrial-embryo development, and implantation. Section 2.1 introduces each of these areas, highlighting the similarities and differences between humans and mice. Section 2.2 unravels an extensive literature review surrounding optimal and suboptimal levels of murine implantation. Attention is drawn towards the lack of standardised *in vivo* implantation murine models and gaps in fundamental knowledge associated with the beginning of endometrial receptivity.

2.1 Background to murine implantation

2.1.1 Endometrial and oocyte development

Human and murine endometrium is a dynamic, nutrient-rich and highly vascular uterine lining, which serves to support embryo growth and development during pregnancy (Diedrich, Fauser, Devroey, & Griesinger, 2007; Tortora & Graboski, 1996; Wang & Dey, 2006). The endometrium consists of two unique layers: 1) the innermost stratum functionalis, which undergoes dramatic degradation and regeneration within each reproductive cycle; and 2) the stratum basalis, which serves as a foundation and proliferates to form the new stratum functionalis (Diedrich, et al., 2007; Tortora & Graboski, 1996).

Interaction of a series of complex negative and positive feedback systems, associated with the hypothalamic-anterior pituitary-gonadal axis, influence endometrial and oocyte development (Hawkins & Matzuk, 2008; Wang & Dey, 2006). In response to low levels of ovarian steroids, estrogen (E) and progesterone (P), hypothalamic gonadotropin releasing hormone (GnRH) stimulates the anterior pituitary to release follicle stimulating hormone (FSH) and luteinizing hormone (LH; Carson, et al., 2000; Psychoyos, 1973b; Tortora & Graboski, 1996). Initially, FSH stimulates ovarian follicular development and release of E and inhibin. Through negative feedback loops, inhibin limits the release of FSH and to a small extent, LH. Reduced levels of FSH cease the development of less mature ovarian follicles. However, the remaining dominant follicles continue to develop within the presence of LH. In addition, P is known to have a non-genomic effect on developing oocytes and the granulosa cells surrounding them. Both express P-receptors, which when bound to P, activates a Ca^{2+}

influx important for meitotic maturation of oocytes (Falkenstein, Tillmann, Christ, Feuring, & Wehling, 2000; McEwen, 1991; Simoncini, & Genazzani 2003). Non-genomic effects of P have also been demonstrated in the sperm capacitation, where P-bound receptors activate Ca^{2+} and Cl^- channels leading to the acrosome reaction (Falkenstein, et al., 2000; McEwen, 1991; Simoncini, & Genazzani 2003).

In humans one or two follicles become dominant (Tortora & Graboski, 1996). In contrast, up to eight to nine follicles, depending on the strain, become dominant within the mouse (Carson, et al., 2000; Hogan, et al., 1994; Psychoyos, 1973b). Dominant ovarian follicles continue to release E and inhibin (Carson, et al., 2000; Psychoyos, 1973b; Tortora & Graboski, 1996). As the level of E continues to rise, the hypothalamus and anterior pituitary are stimulated through positive feedback loops to release high levels of GnRH and LH, resulting in the LH surge. In response, ovulation is triggered. Within humans, ovulation occurs on day-14 of the menstrual cycle, which is approximately 28-days long (Tortora & Graboski, 1996). In contrast, the mice ovulate every four to five days (Hogan, et al., 1994). Nevertheless, humans and mice are both considered spontaneous ovulators (Hogan, et al., 1994; Tortora & Graboski, 1996).

Following the rupture of dominant follicles and oocyte release, LH stimulates follicular transformation into corpus lutea. Subsequently, corpus lutea produce and release predominantly P, but also low levels of E, relaxin and inhibin. Increased levels of P reduce the release of GnRH and LH through negative feedback loops. If fertilization occurs, embryos release chorionic gonadotropin, which supports corpus lutea function. However, in the absence of chorionic gonadotropin, corpus lutea degenerate into corpus albicans, reducing ovarian steroidal secretion. Low levels of P and E stimulate the hypothalamus to increase GnRH secretion and constrict spiral arteries (Totora & Graboski, 1996). The stratum functionalis becomes ischemic and degenerative, and is eventually shed, resulting in menstruation. In contrast to humans, the stratum functionalis is completely reabsorbed within mice (Rudolph, et al., 2012). Thus, starting the reproductive cycle again.

The rudimentary terms of pre-receptive, receptive or refractive can be used to classify endometrial development (Psychoyos, 1973a, 1986; Yoshinaga, 1988). In response to E and P, a number of cytokines, homeobox proteins, and growth factors stimulate the endometrium to undergo substantial genetic, molecular and morphological remodelling, leading to a finite period of receptivity (Dimitriadis, White, Jones, &

Salamosen, 2005; Huet-Hudson, Andrews, & Dey, 1989; Murphy, 2004; Nikas, Develioglu, Toner, & Jones, 1999; Psychoyos, 1973b, 1976; Wang & Dey, 2006). As indicated in Table A2, cytokines, leukaemia inhibitory factor (LIF), interleukin – six (IL-6); homeobox proteins, Hox10a and Hox11a; and transforming growth factor - beta (TGF-β), are important for both endometrial transformation and implantation. The gp130 protein is also essential in endometrial remodelling. When bound to cytokines, the protein dimerizes and initiates the Janus Kinase/Signal Transducer pathway (JAK/STAT; Dimitriadis, et al., 2005; Wang & Dey, 2006).

Once the endometrium is receptive, the implantation-competent embryo has the opportunity to implant. Outside of this period of receptivity, the endometrium acts as a barrier to the developing embryo during both the pre-receptive and refractive phases. The pre-receptive endometrium facilitates a hospitable uterine environment for embryo development, yet, becomes inhospitable during the refractive phase, leading to embryo degradation and loss (Psychoyos, 1976; Yoshinaga, 1988).

Human endometrial receptivity occurs between days 20 to 24 of the menstrual cycle (Hawkins & Matzuk, 2008; Makker & Singh, 2006; Martin, et al., 2002). In contrast, the murine endometrium does not become receptive within the four to five-day estrus cycle, but requires the act of precisely timed coitus and ejaculation following successful ovulation (Allen, 1922; Hogan, et al., 1994; Terkel, 1986; Yang, Larsen, Grattan, & Erskine, 2009). In response, a neuroendocrine reflex stimulates prolactin secretion from the anterior pituitary (Terkel, 1986; Yang, et al., 2009). Murine prolactin supports P secretion from the corpus luteum (CL), which is essential for endometrial receptivity (Terkel, 1986; Yang, et al., 2009). Infertile coitus or manual stimulation, producing pseudo-pregnant mice, can also induce murine endometrial receptivity (Hogan, et al., 1994). The Lee-Boot effect, is an additional phenomenon known to halt estrus and in some cases spontaneously produced pseudo-pregnancy (Van der Lee & Boot, 1955, 1956). Nonetheless, infertile coitus or manual stimulation is considered more efficient. Through the use of pseudo-pregnant mice for ET, the risk of competitive implantation between donor and naïve pre-implantation embryos is eliminated.

It is worth exploring the male factor on murine endometrial receptivity. Multiple studies have linked seminal fluid to endometrial receptivity. Watson, Carroll, and Chaykin (1983) demonstrated a substantial increase in fetal loss and abnormalities following ET into recipient mice that became pseudo-pregnant without the exposure to seminal fluid.

Similar findings were also demonstrated within rats (Carp, Serr, Mashiach, & Nebel, 1984 cited in Robertson, 2005). Fetal growth retardation and poor placental growth were among the observed pregnancy outcomes (Robertson, 2005). Within Robertson's review (2005), it is postulated that seminal fluid prompts an immunological response within the reproductive tract. Possible mechanisms associated include: 1) recruitment of leukocytes involved in endometrial tissue remodelling; and 2), activation of cytokine and growth factor expression (Robertson, 2005).

2.1.2 Embryo development and implantation-competency

Human and murine pre-implantation embryos share a remarkable likeness in regard to their structure and development. Subsequent to fertilization, both species undergo a number of meiotic and cellular divisions, which eventually give rise to the blastocyst embryo (Hogan, et al., 1994; Kirschstein & Skirboll, 2001). Their development and activation is influenced by a number of uterine, and embryonic growth factors and cytokines, in response to E and P (Aplin & Kimber, 2004; Armant, Wang, & Liu, 2000; Carson, et al., 2000). Examples include: calcitonin; heparin binding – epidermal growth factor (HB-EGF); granulocyte-macrophage colony stimulating factor (GM-CSF); insulin-like growth factor 1 (IGF-1); leptin; and leukaemia inhibiting factor (LIF; Table A2, Aplin & Kimber, 2004; Armant,).

Embryonic cells within the blastocyst are polarized and differentiated into the inner cell mass (ICM) and trophectoderm (TD), which surround a fluid-filled cavity, termed the blastocoel (Hogan, et al., 1994; Kirschstein & Skirboll, 2001). Up until this point, the pre-implantation embryo is protected by the zona pellucida (ZP), a highly gylcosylated and sulphated glycoprotein extracellular matrix (Hogan, et al., 1994; Kirschstein & Skirboll, 2001). Blastocoel expansion and two serine proteases, implantation serine proteases 1 and 2, enable the blastocyst embryo to breach its ZP in preparation for implantation, termed hatching (Aplin & Kimber, 2004; Armant, et al., 2000; Guzeloglu-Kayisli, Basar, & Arici, 2007).

Despite the morphological similarities between human and murine pre-implantation embryos, their diameter and cleavage rates differ. The mean diameter of a newly fertilised human embryo is approximately 100 μm (Van Langendonckt, Wyns, Godin, Toussaint-Demylle, & Donnez, 2000). Meanwhile, murine embryos do not reach 100 μm until the blastocyst stage (Ugajin, et al., 2010). The time required for human and murine pre-implantation embryos to become fully expanded blastocysts

also differs (Figure 1). Subsequent to fertilization, approximately the first 54 hours of development are comparable, where both species reach the eight-cell stage (Barlow et al., 1972, and Kiessling & Weitlauf 1979 as cited in Kiessling, Davis, Williams, Sauter, & Harrison, 1991; Wong, et al., 2010). However, human embryos have a slower pre-implantation cleavage rate, ranging from 18 through to 24 hours, dependent on culture conditions (Wong, et al., 2010). In contrast, mouse embryos will cleave on average eight to ten hours, also depending on time of retrieval, culture conditions and mouse strain (Bowman & McLaren, 1970; Barlow et al., 1972, and Kiessling & Weitlauf 1979 as cited in Kiessling, et al., 1991). As a result, *in vitro* cultured mouse embryos reach the expanded blastocyst stage by approximately 96 hours pc (Barlow et al., 1972, and Kiessling & Weitlauf 1979 as cited in Bowman & McLaren, 1970; Harlow & Quinn, 1982; Kiessling, et al., 1991). Meanwhile, human blastocysts only start to appear by 100 hours pc (day-5.0 pc) (Wong, et al., 2010).

In addition to a receptive endometrium, correctly timed embryo implantation-competency is also essential for successful implantation (Singh, et al., 2011; Yoshinaga, 1988). As described above, the exquisite interaction between internal programming of pre-implantation embryos, ZP hatching, and external endometrial cues in the form of secreted and membrane-bound growth factors and cytokines, contribute towards embryo implantation-competency (Table A2; Aplin & Kimber, 2004; Armant, 2005; Armant, Wang, & Liu, 2000; Carson, et al., 2000; Guzeloglu-Kayisli, et al., 2007). In response, protein expression and trafficking of key molecules to the embryonic cell surface increase (Aplin & Kimber, 2004; Armant, 2005; Signh, et al., 2011). Two of which include EGF-receptor and heparin sulfate-proteoglycan (HSPG), which act as early indicators for embryo implantation-competency (Carson, et al., 2000).

It is worth contemplating the source of human pre-implantation embryo knowledge. As previously stated, human data are often restricted to clinical interventions, such as IVF. Frequently, the source of human embryos is associated with both superovulation and *in vitro* culture. Multiple studies have demonstrated adverse effect on the rate of embryo development and blastocyst formation following superovulation of embryo-donor mice (Edwards, Kind, Armstrong, & Thompson, 2005; Ertzeid & Storeng, 1992, 2001; Van der Auwera & D'Hooghe, 2001). Reduced cleavage rates and blastocyst formation, and altered gene expression and metabolic activity have also been associated with mammalian *in vitro* culture (Bowman & McLaren, 1970; Fleming,

et al., 2004; Harlow & Quinn, 1982; Rinaudo & Schultz, 2004; Watkins, et al., 2007). Therefore, it is possible that development of normal human embryo development *in utero* is modulated in comparison to available data.

Again, the male factor is important to consider. Spermatozoal quality can have a major impact on embryo growth, development and subsequent pregnancy outcomes (Chenoweth, 2011; La Rochenbrochard, & Thonneau, 2002; Serre & Robire, 1998). Low sperm quality, which is often linked, but not always, to high paternal age, can result in high pre-implantation embryo loss and low birth weights in rats, rams and bulls (Chenoweth, 2011; Serre & Robire, 1998). In addition, high paternal age has been linked to increased risk of miscarriage in humans (La Rochenbrochard, & Thonneau, 2002). Potential mechanisms leading to poor embryo quality include: 1) chromosomal abnormalities; 2) poor semen viability and morphology; 3) sperm oxidative damage; or 4) environmental factors, for example, increased testicular temperature (Chenoweth, 2011).

Location	Oviduct						Uterus				
Days pc		0.5		1.5		2.5		3.5		4.5	
Hours pc	0	12	24	36	48	60	72	84	96	108	120
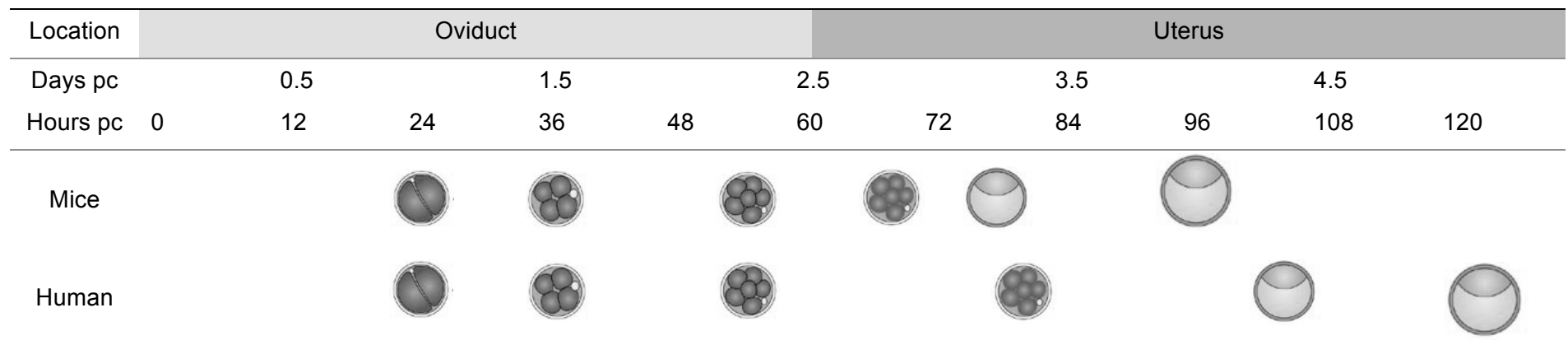
Figure 1 Comparison of mouse and human pre-implantation embryo *in vivo* development. Embryo positions are mean expected times for each stage of development, cross-over can occur. Mouse embryos cleave approximately every ten to twelve hours, depending on strain. Human embryos cleave at least every eighteen hours

2.1.3 Implantation

2.1.3.1. Embryo position and form of implantation

Human and murine embryo positioning is a deliberate process, primarily achieved through uterine muscular contractions (Enders, 1976; Renfree, 1982). However, the final implantation-position differs between the two species (Figure 2). Human embryos are positioned laterally, in the upper two thirds of the uterus, located closest to its fallopian tube of origin (Chavatte-Palmer & Guillomot, 2007; Harper, 1992; Rogers, 1995). Meanwhile, murine embryos implant in an anti-mesometrial position (Renfree, 1982; Rogers, 1995).

The category of implantation also differs between humans and mice. *In vivo* studies within non-human primates suggest that human implantation is interstitial (Renfree, 1982). As seen in Figure 2, the human embryo penetrates the endometrium (Renfree, 1982; Tortora & Graboski, 1996). In contrast, murine implantation is eccentric (Renfree, 1982). Generalised endometrial oedema facilitates murine uterine luminal closure (Psychoyos, 1973a; Renfree, 1982). Consequently, implantation chambers form around each murine embryo (Figure 2).

embryo [TD
        [ICM
lumen
endometrium
mesometrium

A – Human Implantation   **B – Mouse Implantation**
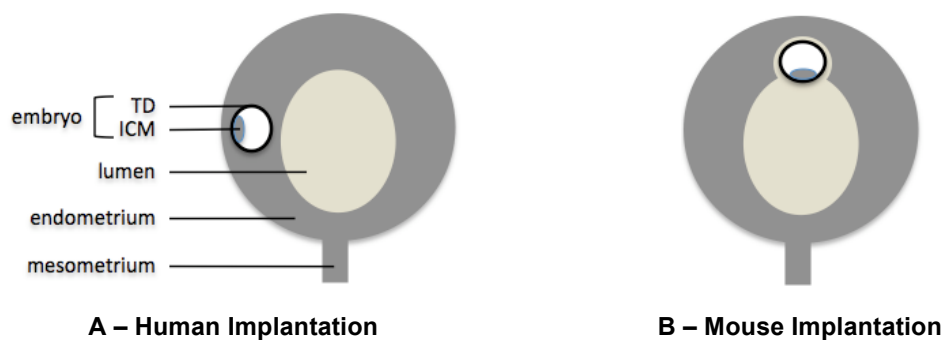
Figure 2 Category, position and orientation of human and murine embryo implantation. Plate A illustrates human interstitial implantation, where trophoblasts invade the endometrium. Embryos are located laterally, with the ICM oriented away from the lumen. Plate B illustrates murine eccentrical implantation, a product of the implantation chamber. Embryos are positioned in an anti-mesometrial fashion, with the ICM oriented towards the mesometrium. Key: TD, trophectoderm; ICM, inner cell mass. Adapted from Bowen (2000).

2.1.3.2. Apposition – the first stage of implantation

Apposition serves three critical objectives: 1) to increase intimate reciprocal embryo-endometrial dialogue; 2) to correctly orient the embryo; and 3) to facilitate the gradual transition into adhesion, the second stage of implantation (Aplin, 2000; Carson,

et al., 2000; Schlafke & Enders, 1975). Both human and murine apposition is achieved through the delicate interaction of trophoblast microvilli and epithelial uterodomes (humans) or pinopodes (mice) (Norwitz, 2006; Wang & Dey, 2006). As seen in Figure 2, such interaction enables correct embryo orientation, where the human ICM orients towards the uterine wall, and the murine ICM orients towards the mesometrium (Aplin, 2000; Armant, 2005; Schlafke & Enders, 1975).

One of the first indicators of apposition and embryo-maternal cross-talk is the expression of HB-EGF on luminal endometrial epithelia, and ErbB4 expression on embryonic trophectoderm (Dey & Wang, 2006; Fukuda & Sugihara, 2008). Loose lectin-to-glycan and glycan-to-glycan binding (embryo-to-endometrium) has also been associated with initial apposition (Aplin, 1997, 2006). Two unique mechanisms of action associated with human apposition include L-selectin, and trophinin-bystin-tastin binding complexes (Fukuda & Nozawa, 1999; Fukuda & Sugihara, 2008).

The carbohydrate-binding group of selectins, E, P and L-selectin, are involved in both human and mouse immunological processes (Fukuda & Sugihara, 2008; Genbacer, et al., 2003). L-selectin is of particular interest, where it's expression in humans and non-human primates includes blastocyst trophectoderm (Fukuda & Sugihara, 2008; Genbacer, et al., 2003). In contrast, L-selectin expression is absent from murine blastocysts.   Outside of human trophectoderm, L-selectin is expressed on lymphocytes, and is involved in their recruitment for immunological response. It has been postulated that L-selectin plays a similar role in implantation, where blastocysts slowly roll to their place of implantation (Fukuda & Sugiara, 2008; Genbacer, et al., 2003).

Trophinin-bystin-tastin complexes replace the initial L-selectin binding, and are also unique humans and non-human primates (Fukuda, & Nozawa, 1999; Fukuda & Sugihara, 2008). Trophinin is expressed on both endometrial epithelia and blastocyst trophectoderm at the time of implantation (Carson, et al., 2000; Fukuda & Sugihara, 2008). Alone, trophinin cannot bind to itself, but requires the cytoplasmic proteins, bystin and tastin. Together, these proteins form a complex enabling homophilic cell adhesion (Carson, et al., 2000).

2.1.3.3. Adhesion – the second stage of implantation

Extensive morphological and molecular modifications are observed throughout human and murine adhesion, the second stage of implantation. In context of morphology,

trophoblast proliferation increases dramatically, resulting in their differentiation into syncytial and cytotrophoblasts (Bazer, Spencer, Johnson, Burghardt, & Wu, 2009; Enders, 2000; Tortora & Graboski, 1996). Increased stromal vascular permeability at the implantation site is also observed (Bazer, et al., 2009; Dey, et al., 2004; Psychoyos, 1961). Combined with the intravenous administration of blue-dye prior to autopsy, increased vascular permeability can be exploited to detect the first gross indication of implantation (Psychoyos, 1961).

Substantial molecular profile transformation facilitates the firm adhesion of implantation-competent embryos to endometrial epithelium (Table A1 and Table A2; Achache & Revel, 2006; Aplin, 2006; Armant, 2005; Dey, et al., 2004; Fukuda & Sugihara, 2008; Wang & Dey, 2006). In context of the embryo, gene expression, protein turn-over, glycosylation and protein trafficking mediate this adhesion (Aplin, 1997; Carson, et al., 2000; Wang & Dey, 2006). Comparatively, endometrial paracrine signalling, and simple cellular recognition at the trophectoderm surface, invokes specific gene transcription of trophectoderm receptors (Armant, 2005; Dey, et al., 2004). For example, endometrial epithelium H-type-1, a component of MUC-1, binds with trophoblast expressed Le^Y, forming a carbohydrate-carbohydrate interaction (Aplin, 1997, 2000; Aplin & Kimber, 2004).

2.1.3.4. Invasion – the final stage of implantation

Although the purpose of both human and murine invasion is for trophoblasts to become more intimately associated with the endometrium, their strategies differ. Human invasion involves the intrusion of syncytial trophoblasts between endometrial epithelial cells, whose intracellular junctions have diminished by this time (Aplin, 2000; Aplin, Haigh, Lacey, Chen, & Jones, 2000; Bazer, et al., 2009; Renfree, 1982). Intrusion is achieved by a number of integrin-to-ECM component interactions and proteases (Bazer, et al., 2009; Chavatte-Palmer & Guillomot, 2007). For example, binding between trophectoderm $\alpha 5 \beta 1$ to fibronectin initiates migration through ECM (Chavatte-Palmer & Guillomot, 2007). Subsequently, trophoblast derived metalloproteases and serine proteases help breakdown the ECM (Chavatte-Palmer & Guillomot, 2007). In contrast, trophoblasts within murine invasion displace endometrial epithelial cells by phagocytosis (Chavatte-Palmer & Guillomot, 2007; Enders, 1976; Renfree, 1982). Thus, invasion is much more superficial compared to humans (Chavatte-Palmer & Guillomot, 2007).

Transformation of stroma into decidua, termed decidualization, is essential for continued support of pregnancy. The extent of decidualization is dependent on fetal:placental nutrient demand, which often reflects the degree of invasion (King, 2000). Within humans, the beginning of decidualization occurs automatically within the menstrual cycle, independent of embryo implantation (King, 2000). In response to P following ovulation, dramatic changes occur within endometrial stroma. These include: 1) differentiation of stromal cells into decidua, five-times their original size; 2) glandular tissue becomes attenuated and non-secretory; 3) levels of uterine natural-killer cells increase substantially; and 4) spiral arteries elongate, become increasingly convoluted and endothelial cell walls thicken (Carter, 2007a; King, 2000). If implantation is successful, maternal vasculature is modified by the degradation of endothelial cells and replacement by invading syncytial trophoblastic cells, a feature of hemochorial placentation (Bazer, et al., 2009).

In contrast to humans, mice require embryo implantation as a stimulus for decidualization (Das, 2009; Tan, Paria, Dey, & Das, 1999). For this reason, artificial stimulus can trigger decidualization with pseudo-pregnant or ovarian steroid primed mice (Das, 2009). As stated previously, mouse embryo invasion is considered superficial compared to humans (Chavatte-Palmer & Guillomot, 2007). Following implantation, on day-4.5 pc, stroma immediately surrounding the implanted embryo proliferates and differentiates into the primary decidual zone (PDZ; Das, 2009; Tan, et al., 1999). Decidua is densely packed and lacks vasculature (Das, 2009). By day-5.5 pc, the subsequent layer of stroma also begins to proliferate and differentiate, forming the secondary decidual zone (SDZ; Das, 2009; Tan, et al., 1999). By this time, proliferation has ceased within the PDZ. Syncytial trophoblastic cells begin to invade spiral arteries within the SDZ, but do not extend past this zone (Carter, 2007a). By day-7.5 pc onwards, embryo and placental growth slowly replaces the SDZ (Das, 2009).

2.2 *In vivo implantation murine modelling*

2.2.1 Need for *in vivo* animal modelling

Implantation is crucial for successful pregnancy, yet little understanding surrounds this pivotal step. Ethical and legal restrictions associated with human research contribute towards lack of implantation knowledge (Deech & Smajdor, 2007; Hannan, Paiva,

Dimitriadis, & Salamonsen, 2010; Lee & DeMayo, 2004; Rogers, 1995; Singh, Chaudry & Asselin, 2011). Alternatively, *in vitro* and *in vivo* implantation modelling provide controlled experimental frameworks (Carson, et al., 2000; Lee, Jeong, Tsai, Lydon, & DeMayo, 2007; Singh, Nardo, Kimber, & Aplin, 2010; Wang & Dey, 2006). While human and animal *in vitro* implantation modelling can extrapolate the intimate details of human implantation, in the absence of human experimentation, only *in vivo* animal modelling can provide an *in utero* perspective. However, none are recognized as standard and thus limit their contribution towards implantation knowledge.

Many factors bring reason to the ethical and legal constraints surrounding human research. Although human *in vivo* studies could help elucidate implantation, such exploration is considered too invasive, placing the fetus at undue risk (Deech & Smajdor, 2007). The random allocation of experimental treatments, without considering the impact on pregnancy success, could also be difficult to rationalise. Moreover, human subjects would present their own unique set of variables based on health, nutrition and lifestyle (ASRM Practice Committee, 2006; Bontekoe, Blake, Heineman, Williams, & Johnson, 2010; Van Voorhis & Dokras, 2008; Wolner-Hanssen & Rydhstroem, 1998). Such variability could compromise subject comparability within treatment groups.

Alternatively, researchers have exploited tools associated with clinical interventions to illuminate human implantation. While clinical interventions, such as human-IVF, facilitate a convenient source of data, information gathered is somewhat limited. Four reasons contribute towards this limitation. Firstly, data are indirectly correlated with human implantation (Aghajanova, Hamilton, & Giudice, 2008; Arnold, Kaufman, Seppala, & Lessey, 2008; Makker & Singh, 2006). For example, endometrial biopsy and ultrasound use histological dating, endometrial thickness and pattern to estimate the time of endometrial receptivity, the finite period of time the endometrium allows embryo implantation (Aghajanova, et al., 2008; Coutifaris, et al., 2004; Jayaprakasan, et al., 2010; Makker & Singh, 2006). Secondly, data associated with clinical interventions is often reported retrospectively rather than through randomised controlled studies (De Mouzon, et al., 2010; Wang, Chambers, Dieng, & Sullivan, 2009). Potential bias from multiple experimental variables is therefore difficult to eliminate and reduces comparability between studies. Thirdly, data can be limited by incomplete representation of the entire populous (De Mouzon, et al., 2010; Wang, et al., 2009). Couples that have impaired fertility typically utilize clinical interventions.

Consequently, data may not fully elucidate implantation within normal fertile women. Fourthly, discrepancies surrounding accuracy and reproducibility of the available tools limit data (Aghajanova, et al., 2008; Jayaprakasan, et al., 2010; Makker & Singh, 2006).

Two-dimensional (2D) and three-dimensional (3D) *in vitro* implantation modelling enable both close examination of trophectoderm – endometrial epithelial cell interaction and increased controlled scientific experimentation (Bentin-Ley & Lopata, 2000; Mardon, Grewal, & Mills, 2007). Two-dimensional *in vitro* implantation modelling, involves the attachment challenge of embryos or spheroids (aggregates of embryonic trophectoderm) to endometrial epithelial cell monolayers (Heneweer, et al., 2002; Kosaka, et al., 2003; Li, et al., 2002; Shiotani, Noda, & Mori, 1993). Such attachment is challenged by centrifugation, rotation or gyration. For example, Williams (2008) presents a potential *in vitro* attachment assay, where FSL-HA manipulated embryos were incubated with human endometrial epithelial RL95-2 cell monolayers and compared with unmanipulated embryos (Table 39). Attachment was challenged by inversion and centrifugation. In contrast, construction of 3D *in vitro* implantation modelling has greater sophistication, more closely resembling *in utero* (Arnold, et al., 2001; Bentin-Ley & Lopata, 2000; Bentin-Ley, et al., 1994; Park, et al., 2003). Nonetheless, *in vitro* modelling cannot provide the detailed *in utero* perspective that is possible within *in vivo* animal modelling.

Numerous species offer the opportunity to study implantation *in vivo*. Non-human primates offer the most analogous comparison to humans (Carson, et al., 2000; Enders, 2000; Lee & DeMayo, 2004). Not only is their genomic profile most comparable to humans, but also their simplex slot-like uterus (Lee & DeMayo, 2004). Yet, the low experimental output and high experimental cost make non-human primates a challenging species to work with (Lee & DeMayo, 2004). While alternative species are not strictly analogous to human implantation, their specific characteristics enable detailed investigation into different stages of implantation (Carson, et al., 2000; Enders, 2000; Lee & DeMayo, 2004). One such example is the pig, whose apposition and adhesion stages of implantation is extended and permits thorough examination of early implantation (Lee and DeMayo 2004).

Mice offer a further alternative to non-human primates. Several features account for the viability of *in vivo* implantation murine modelling. The first three features surround murine physiology. Murine ovulation firstly occurs every four to five days (Hogan,

et al., 1994). In contrast, non-human primate ovulation can occur every 15 – 28 days, depending on the species (Garcia, Shimizu, & Huffman, 2009; Sommer, Srivastava, & Borries, 1992; Ziegler, et al., 1993). As consequence, the opportunity for successful murine pregnancy or pseudo-pregnancy occurs up to seven times more often than in non-human primates. Secondly, murine gestation is 19 – 21 days long, followed by only 40 days to become sexually mature (Hogan, et al., 1994). Meanwhile, the gestation length of most non-human primate species exceeds 130 days and years to reach sexual maturity (Sommer, et al., 1992; Wilson, Gordon, Rudman, & Tanner, 1989). Thirdly, most non-human primates sustain singleton live births (Cowlishaw, et al., 2007). Mice, however, are polytocous by nature, where multiple live young are supported (Hogan, et al., 1994; Renfree, 1982). The polytocous nature of mice could be considered a negative feature when studies attempt to translate findings directly into the human clinical situation. However, rather than establishing standard *in vivo* implantation murine models that closely reflected human IVF, this research focused on understanding improved standardization of models suitable to explore implantation and preliminary understanding of IVF-intervention potential. For this reason, in comparison to non-human primates, the substantially higher experimental output exhibited by mice was considered favourable.

Three more features contribute towards the viability of *in vivo* implantation murine modelling. The space, time and expense required to house murine colonies are substantially less than non-human primates (Carson, et al., 2000; Lee & DeMayo, 2004). Thus the overall experimental cost is considerably reduced. The techniques associated with murine implantation research are also well established. Some of which include embryo retrieval, ET, or intravenously injected blue dye for the first gross detection of implantation sites and decidual responses (Hogan, et al., 1994; Psychoyos, 1961, 1973a). In addition, the extensively researched murine genome provides an effective platform to explore molecular and functional studies surrounding the early stages of implantation (Carson, et al., 2000; Lee & DeMayo, 2004; Lee, et al., 2007).

Despite the vast array of *in vivo* implantation murine models available, none are recognised as standard. Three reasons, revealed by an extensive literature review, appear to contribute towards the lack of standardization. Firstly, mouse-to-mouse variability within individual embryo transfer studies is difficult to ascertain. Secondly, implantation rates between different research groups, which appear to utilize

comparable models, are inconsistent. Thirdly, the impact of altered embryo-endometrial developmental synchrony on the timing of endometrial receptivity and ultimately implantation rates has not been meticulously explored. As a consequence, comparison between different research groups can be difficult. Sections 2.2.2 and 2.2.3 will serve to examine these reasons in greater depth, which will include a comprehensive background into *in vivo* implantation murine modelling.

2.2.2 Current *in vivo* implantation murine models

To begin to understand improved standardization of *in vivo* implantation murine models, suitable to explore implantation and preliminary studies surrounding IVF-intervention potential, an extensive literature review was performed. Attention was directed towards elucidating existing knowledge surrounding optimal and suboptimal murine implantation. In context of this research, the terminology optimal and suboptimal was used to describe the level of implantation, not the quality of each implant. It was necessary to differentiate levels of implantation as they offered different insights into IVF-intervention potential.

The term optimal was applied to implantation rates that were comparable or greater than natural mouse pregnancy (strain dependent). Within *in vivo* implantation murine models that exhibited optimal levels of implantation, any detrimental effect of an IVF-intervention could be detected by reduced implantation rates. Thus, determining the preliminary safety of that intervention. In contrast, implantation rates that were ≤70% of natural mouse pregnancy were considered suboptimal (also be strain dependent). Models that achieved suboptimal implantation rates could be used to assess the ability of an IVF-intervention to increase implantation level, which could translate to improved live birth rates. A maximum level of implantation was set at 70% of natural mouse pregnancy as it could easily demonstrate significant increases in implantation rates, but also keep numbers of experimental mice to a minimum. Chapter 4 will expand these terms in greater detail.

Within this research, specific selection criteria will be applied to each term, and will be used to interpret baseline data of each *in vivo* implantation murine model explored. Depending on the criteria met, models will be categorised as optimal or suboptimal. The most suitable of these models will be selected and assessed for their ability to detect consequences of manipulated embryo, and IVF-intervention (Sections 4.5 and 4.6). It is important to note, that while a model may present a level of implantation

unsuitable to explore initial IVF-intervention potential, it may be more than suitable to explore another area of research. For example, knock-out mice, which typically result in eliminated rather than reduced implantation levels, are highly advantageous for functional studies surrounding implantation molecules (Song, Lim, Das, Paria, & Dey, 2000; Tranguch, Chakrabarty, Guo, Wang, & Dey, 2007).

The next step within the literature review was to identify potential *in vivo* implantation murine models suitable for different levels of implantation. The focus was placed on models associated ET, rather than the entire IVF procedure. Coupled with superovulated donor embryos, ET required less time and technical skill compared to IVF. Thus greater flexibility and experimental output were enabled.

In addition to ET, the descriptors of modelling, mice, implantation, fertile and sub-fertile were included within the literature search. Ninety studies, describing nine different approaches to generate ET models, were revealed. While it was important models were suitable for preliminary studies surrounding IVF-intervention potential, it was not crucial that they held direct relevance to clinical scenarios. Nonetheless, each approach mimicked a different aspect of human and animal reproductive interventions, and could be broadly categorized into two groups (Table 1 and Table 2).

The first category encompassed recipient mice that did not require an external manipulation outside of ET. Mice that were deficient in an implantation-related gene were one such example (Fouladi-Nashta, et al., 2005; Song, et al., 2000; Tranguch, et al., 2007; Varani, et al., 2002; Ye, et al., 2005). The second category incorporated recipient mice that did require an external manipulation outside of ET. For example, ET into superovulated recipient mice. In this instance, superovulatory drugs were administered prior to ET, which led to an advanced endometrium, reducing implantation opportunity (Ertzeid & Storeng, 2001; Kelley, et al., 2006; Ma, Song, Das, Paria, & Dey, 2003). As this research identified a lack of clarity surrounding the impact of altered embryo-endometrial developmental synchrony on implantation rates, only studies, which utilised models that could explore this subject, were included. Twenty-one studies remained as a result.

Model standardization would strengthen experimental data; enable greater comparison between research groups and aid development of effective research platforms, useful to explore implantation and preliminary studies surrounding IVF-intervention potential. For example: 1) investigating whether IVF-interventions were detrimental to ET

outcomes; and 2) could that intervention restore suboptimal implantation rates to normal levels. Therefore, the overall research objective was to gain insight into improved standardization of *in vivo* implantation murine models that were suitable for the purposes specified above.

Three different approaches were investigated in greater detail. These included: 1) ET into superovulated recipient mice (advanced endometrium); 2) progesterone antagonist administration following ET (delayed endometrium); 3) synchronous and asynchronous ET (donor embryo and recipient mouse endometrium developmental age purposefully shifted at the time of transfer). It is important to note that the extent of endometrial development was simply used to quickly describe each model. The embryo was equally important to consider, and is described in more detail for each model in Chapters 4 and 5.

Table 1 Examples of *in vivo* implantation murine models that do not require an additional manipulation of recipient mice to generate the model.

Model	Impact	Mimicked procedure	Limitations	References
Synchronous ET	Developmental age (days pc) of embryo and recipient mouse endometrium is equal	Human embryo donor or agricultural ET	Not truly synchronous when donor embryos are cultured *in vitro* prior to ET.	Cheng, et al., 2004; Doyle, Gates, & Noyes, 1963; Goto, et al., 1993; McLaren & Michie, 1955; Mullen & Carter, 1973; Nakayama, et al., 1995; Paria, Huet-Hudson, & Dey, 1993; Song, Han, & Lim, 2007; Wakuda, et al., 1999; Wiebold & Anderson, 1986
Asynchronous ET	Embryo developmental age (days pc) less or more than recipient mouse endometrium	Frozen human embryo IVF cycle		
Older mice (> 120 days old)	Less effective, older endometrium. Uterine environment possibly less hospitable to donor embryos.	Average age of women undergoing assisted reproduction technology (ART) in Australia and New Zealand is 35.8 years (2009 ART Statistics).	Mice ovulate less often. Therefore, high numbers of experimental mice required.	Munne, Alikani, Tomkin, Grifo, & Cohen, 1995; Wang, Macaldowie, Hayward, Chambers & Sullivan, 2011
Knock-out mice	Deficiency in a specific gene	Good for studying molecules potentially involved in implantation.	Implantation is typically eliminated rather than reduced. Alternatively, molecular redundancy ensures implantation is successful despite the deficiency. High monetary expenditure involved.	Fouladi-Nashta, et al., 2005; Song, et al., 2000; Tranguch, et al., 2007; Varani, et al., 2002; Ye, et al., 2005; Paria, Song, & Dey, 2001
Transgenic mice	Insertion of gene from a different organism, which can induce overexpression of gene.	Good for studying proteins or enzymes important in endometrial remodelling and implantation.	High monetary expenditure involved.	Paria, et al., 2001; Alexander, et al., 1996

Table 2 Examples of *in vivo* implantation murine models that require an additional manipulation of recipient mice to generate the model.

Model	Impact	Mimicked procedure	Limitations	References
Superovulatory drugs (FSH and LH; without luteal/progesterone support)	Indirectly advances endometrium through stimulating the secretion of ovarian steroids (P and E). Uterine environment can be less hospitable to donor embryos. Reduced implantation and intrauterine growth retardation result.	Fresh human IVF cycles without luteal support	Requires high experimental mice numbers and complex planning	Blake, 1997; Bourgain & Devroey, 2003; Ertzeid & Storeng, 1992, 2001; Fanchin, De Ziegler, Taieb, Hazout, & Frydman, 1993; Fossum, Davidson, & Paulson, 1989; Gidley-Baird, et al., 1986; Kelley, et al., 2006; Lee, et al., 2008; Ma, et al., 2003; Ng, Yeung, Lau, So, & Ho, 2000; Oehninger, 2008; Ronnberg, Isotalo, Kauppila, Martikainen, & Vihko, 1985; Safro, O'Neill, & Saunders, 1990; Sibug, et al., 2005
Progesterone antagonists (i.e. RU486)	Slows endometrial development by blocking P-receptor and inhibiting mitotic division.	Contragestive intervention, which prevents implantation (increased pre-implantation loss).	Can also be abortive (increased post-implantation loss). Action and extent of influence is administration time and dose dependent.	Cameron, et al., 1996; Croxatto, et al., 1998; Gemzell-Danielsson, Mandl, & Marions, 2003; Gemzell-Danielsson, Swahn, Svalander, & Bygdeman, 1993; Huang, Nardo, Huang, Lu, & Liu, 2005; Jain, Li, Yang, Minoo, & Felix, 2007; Lalitkumar, Sengupta, Karande, & Ghosh, 1998; Li, Felix, Minoo, Amezcua, & Jain, 2005; Liu, Huang, Lu, Gong, & Zhang, 2003; Sarantis, Roche, & Psychoyos, 1988
High levels of exogenous estrogen	Directly accelerates endometrial development	Fresh human IVF cycles	Requires high experimental mice numbers and complex planning	Ma, et al., 2003; Ng, et al., 2000
Transient liposome-mediated plasmid transfection (transient knock-down)	Temporary blockage of specific messenger ribonucleic acid. Trans-vaginal delivery prior to ET	Good for studying molecules potentially involved in implantation	Expensive and difficult to generate stable plasmids	Daftary & Taylor, 2003; Nakamura, et al., 2006; Wadehra, et al., 2006; Zhuo, et al., 2001; Paria, et al., 2001

Embryo transfer into superovulated (SO) recipient mice, without progesterone support, is thought to advance endometrial development (Kelley, et al., 2006; Oehninger, 2008). A number of observations throughout literature are indicative of such advanced development. One example is the advanced molecular profile of superovulated murine endometrium compared to its real-time age (Kelley, et al., 2006; Ma, et al., 2003; Sibug, Helmerhorst, Tijssen, de Kloet, & de Koning, 2002). This can include: a) the premature reduction in P and E-receptor expression; and b) reduced integrin expression (Bourgain & Devroey, 2003; Fanchin, et al., 1993; Lee, et al., 2008; Ng, et al., 2000; Oehninger, 2008; Ronnberg, et al., 1985). The early appearance of uterodomes (human) or pinopodes (mice) also provides morphological evidence of advanced endometrial development. Moreover, literature demonstrates reduced endometrial perfusion within superovulated endometrium, which also signifies advanced development. Ultimately, a number of human and murine studies suggest that altered serum E:P ratio could be responsible for the advanced endometrial development observed (Bourgain & Devroey, 2003; Lee, et al., 2008; Ng, et al., 2000; Ronnberg, et al., 1985).

Administration of progesterone antagonists, such as RU486, can elicit an abortive, contragestive or contraceptive effect *in utero*. Mifepristone (RU486) binds to the P-receptor with higher affinity than P or P-agonists (Cadepond, Ulmann & Baulieu, et al., 1997; Spitz, 2003). While binding activates dimerization of the P-receptor, association with progesterone response elements is unproductive. In response, corepressors are enlisted, and DNA transcriptional activity is suppressed (Capepond, et al., 1997; Spitz, 2003). However, dosage level determines whether RU486 has an abortive, contragestive or contraceptive effect. While high dosages of RU486, in combination with prostaglandins, are accepted as an effective second trimester abortificant, low dosages of RU486 can be used as a contragestive or contraceptive ("Antiprogestogens: from abortion to contraception," 1992; Cadepond, et al., 1997; Cameron, et al., 1996; Gemzell-Danielsson, et al., 2003; Gemzell-Danielsson & Marions, 2004; Ghosh, Kumar, & Sengupta, 1997; Mahmood, Saridogan, Smutna, Habib, & Djahanbakhch, 1998; B. Yang, Zhou, He, & Fang, 2000).

Human and murine studies indicate that a number of mechanisms of action are associated with low doses of RU486. These can include: 1) delayed ovulation; 2) altered tubal function; 3) inhibited pre-implantation embryo development; 4) repressed endometrial mitoses; and 5) reduced embryonic invasion (Dai, et al., 2003;

Gemzell-Danielsson, et al., 2003; Gemzell-Danielsson & Marions, 2004; Ghosh, Lalitkumar, Wong, Hendrickx, & Sengupta, 2000; Huang, et al., 2005). However, which mechanism of action operates is dependent on the time of administration ("Antiprogestogens: from abortion to contraception," 1992; Cadepond, et al., 1997).

Within this body of work, the subcutaneous administration of RU486 into pseudo-pregnant recipient mice one-day post-ET (day-3.5 pc) was thought to delay endometrial development (Cameron, et al., 1996; Croxatto, et al., 1998; Sarantis, et al., 1988). Possible alteration to ovulation and tubal function was not considered a concern, as these were associated with earlier ET or natural mouse pregnancy (Gemzell-Danielsson, et al., 2003; Gemzell-Danielsson & Marions, 2004; Huang, et al., 2005; Mahmood, et al., 1998). Inhibited blastocyst development and viability were also unlikely, as donor embryos were produced and cultured within an environment lacking RU486 (Lalitkumar, et al., 2007). However, RU486 administration could lead to delayed endometrial development through a number of mechanisms. These included: inhibited endometrial mitoses, by halting the cell cycle at the S phase; delayed pinopode development; reduced expression of Fas and FasL genes, thus reducing apposition at the implantation site; and reduced embryo invasion (Gao, Xu, Zhou, Han, & Liu, 2001; Huang, et al., 2005; Li, et al., 2005; Liu, et al., 2003; Liu, Huang, Yang, & Lu, 2008; Sarantis, et al., 1998). For these reason, delayed endometrial development was considered one of the primary mechanisms of action within this research.

Synchronous and asynchronous ET facilitates a simple experimental platform utilised within many different species. No additional manipulation of recipient animals, outside of ET, is required. At the time of transfer, embryo-endometrial developmental synchrony is purposefully shifted at the time of ET (McLaren & Michie, 1955; Song, et al., 2007; Wakuda, et al., 1999). As a result, more or less opportunity for implantation is created. Synchronous ET is where the developmental age of donor embryos and recipient mouse endometrium are equal (McLaren & Michie, 1955; Noyes, Dickmann, Doyle, & Gates, 1963). In contrast, asynchronous ET is where the developmental age of donor embryos is more or less than the recipient mouse endometrium (Doyle, et al., 1963; McLaren & Michie, 1955). How the alteration of embryo-endometrial developmental synchrony impacts optimal and suboptimal implantation rates is somewhat unclear and is discussed in greater depth within Section 2.2.3.

In addition to mice, synchronous and asynchronous ET has been explored within many other species. Their findings help identify which scenarios produce the best pregnancy outcomes. Studies within cattle, rabbit and sheep have demonstrated little difference between synchronous and asynchronous ET, where embryo developmental age was comparable, older or younger than the recipient endometrium (Adams, 1980; Wilmut, Ashworth, Springbett, & Sales, 1988; Wilmut, Sales, & Ashworth, 1985). For this reason, synchronous ET are normally adopted within these species. In contrast, pregnancy outcomes were substantially improved within rats and pigs when donor embryo developmental age was comparable or older than recipient endometrium (Dickmann and Noyes, 1960; Wilmut, et al., 1985). Those donor embryos that were less developed often resulted in failed or low implantation. Studies also observed decreased or eliminated implantation following very early or late synchronous ET within rabbits, rats and pigs (Adams, 1980; Dickmann & Noyes; 1960; Webel, Peters, & Anderson, 1970). Data suggested that there was a limited period of time where synchronous ET would result in successful pregnancy outcomes. In addition, if the asynchrony between donor embryos and recipient endometrium extended further than two days within sheep, reduced or eliminated implantation rates were observed (Wilmut, et al., 1985).

2.2.3 Lack of standardised models

Although four approaches were identified, none were recognised as standard *in vivo* implantation murine models. Five reasons contributed towards the lack of standardization. Firstly, mouse-to-mouse variability within individual studies was difficult to determine. Secondly, inconsistent implantation rates were observed between studies. The third and fourth reasons surrounded an understanding of normal murine pregnancy. Not only did the beginning of murine endometrial receptivity lack clarity, but also the impact of altered embryo-endometrial developmental synchrony on the timing of that period and implantation rates was also unclear. The fifth reason surrounded experimental design. Often the overall research objective of each study differed. Their experiments were, therefore, designed to achieve their research objective rather than focusing on standardization. Nonetheless, without understanding normal murine implantation, the strategies used to establish consistent models were somewhat arbitrary.

It is also important to note, that within this section and subsequent sections, real-time development of donor embryos and recipient mouse endometrium is described as E"x"

and R"x" days pc. While the letters E and R represent the donor embryo and recipient mouse endometrium, x represents the number days pc. In addition, positive or negative symbols are added to describe any delayed or advanced development in comparison to real-time. For example: 3.5-days following coitus an *in vitro* cultured embryo, whose development is slightly delayed compared to natural mouse pregnancy, is described as a E3.5(-) embryo. In contrast, a recipient mouse whose endometrium has matured for 2.5-days following coitus is described as a R2.5 recipient mouse.

2.2.3.1. Mouse-to-mouse variability within studies is difficult to determine

Within this extensive literature review, the majority of studies expressed implantation rates as a percentage frequency of the total numbers of embryos transferred rather than the mean or individual numbers of implants per recipient mouse (Goto, et al., 1993; Song, et al., 2007; Wakuda, et al., 1999). Although percentages did enable easier comparison between studies, statistical analysis that could indicate model consistency, such as standard deviation, was often not reported (Cheng, et al., 2004; Ertzeid & Storeng, 2001; Goto, et al., 1993; Song, et al., 2007; Wakuda, et al., 1999). Therefore, it was difficult to determine mouse-to-mouse variability within studies.

2.2.3.2. Inconsistent implantation rates between research groups

Implantation rates varied substantially between studies that appeared to utilize comparable *in vivo* implantation murine models (Chapter 9, Table A3). Figure 3 illustrates such variation when implantation rates following synchronous and asynchronous ET were compared between ten different published studies. Six different *in vivo* implantation murine models were created subsequent to the transfer of E3.5 embryos into R0.5 through to R5.5 recipient mice. Although the real-time embryo-endometrial developmental synchrony of each model was comparable between research groups, implantation rates varied up to 68% (McLaren & Michie, 1955; Rulicke, Haenggli, Rappold, Moehrlen, & Stallmach, 2006).
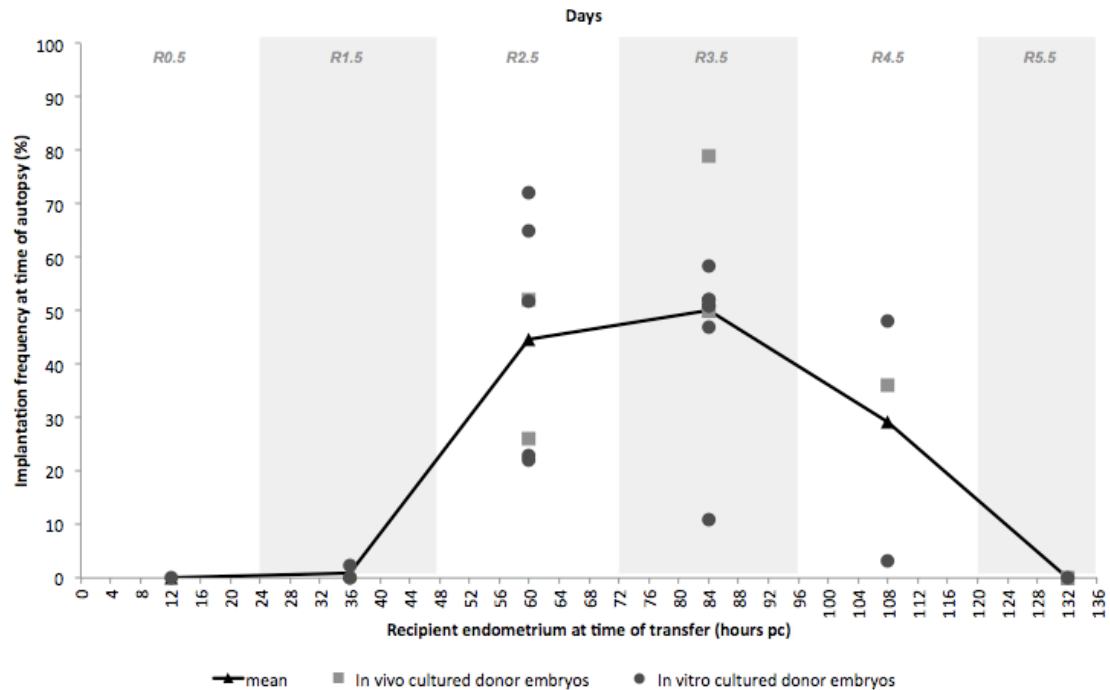
Figure 3 Implantation rates of six different synchronous and asynchronous ET models compared between ten published studies. The transfer of E3.5 embryos into R0.5 through to R5.5 recipient mice created each model. Implantation rates were expressed as a percentage frequency of the total numbers of embryos transferred. Data, representing at least 270 ET, referenced from Fossum, et al., 1989; Goto, et al., 1993; Lane & Gardner, 1994; Lim, et al., 2006; McLaren & Michie, 1955; Mullen & Carter, 1973; Paria, et al., 1993; Rulicke, et al., 2006; Song, et al., 2007; Wakuda, et al., 1999.

Detailed analysis, presented in Table 3, demonstrated numerous experimental variables between studies. For example, at least seven experimental variables differed between those research groups who transferred E3.5 embryos into R3.5 recipient mice. Some of which included: the numbers and strain of recipient mice; time of assessment; age and superovulation-status of embryo donor mice; *in vitro* culture status of donor embryos; specific media and culture conditions; and numbers of donor embryos transferred (Fossum, et al., 1989; Goto, et al., 1993; McLaren & Michie, 1955; Paria, et al., 1993; Rulicke, et al., 2006; Wakuda, et al., 1999). In fact, there were a multitude of potential experimental variables surrounding donor embryos (Figure 4), recipient mice (Figure 5), and the ET itself (Figure 6). Over 800 different experimental scenarios resulted when Figures 4 to 6 were combined. This number increased further when technical skill and quality of surgical equipment were considered. Mouse colony environmental conditions were also likely to differ between research groups. Such conditions could have included: a) ratio of daylight to darkness within any 24-hour period; b) male to female colony ratios; c) levels of natural estrogens within diet; and d) the time of year (Hogan, et al., 1994). As a result, data between different research groups was somewhat incomparable.

Table 3 Numerous experimental variables present within data, illustrated in Figure 3. Key: SO, superovulated; N, naturally ovulated; and U, urinary.

Researcher	Implantation rate (%)	Number of recipients	Recipient mouse strain	Time of assessment (days pc)	Age of embryo donor mouse	Naturally or superovulated	Urinary or recombinant gonadotropin	Day of embryo retrieval	Embryo culture status	Number of embryos transferred per recipient	Number of embryos transferred per uterine horn
Model: Asynchronous ET, E3.5/R0.5											
Wakuda, et al., 1999	0	20	ICR	11	adult	SO	U	3.5	*in vivo*	6	3
Model: Asynchronous ET, E3.5/R1.5											
Wakuda, et al., 1999	0	20	ICR	11	adult	SO	U	3.5	*in vivo*	6	3
Goto, et al., 1993	2	12	ICR	11.5	adult	SO	U	3.5	*in vivo*	5 - 7	-
Model: Asynchronous ET, E3.5/R2.5											
McLaren & Michie, 1955	22	15	C3H x C57BL	16.5	prepub	SO	U	3.5	*in vivo*	mean of 9	-
McLaren & Michie, 1955	23	16	C3H x C57BL	16.5	prepub	SO	U	3.5	*in vivo*	mean of 9	-
Lim, et al., 2006	26	13	ICR	4.5	adult	SO	U	2.5	*in vitro*	20	10
Wakuda, et al., 1999	52	20	ICR	11	adult	SO	U	3.5	*in vivo*	6	3
Lane & Gardner, 1994	52	-	C57BL/6 x CBA/Ca	15	prepub	SO	U	0.5	*in vitro*	-	-
Mullen & Carter, 1973	65	12	C57BL/10 GnDg	16 - 17	-	N	-	2.5	*in vitro*	8 – 14	-

29

Mullen & Carter, 1973	72	12	(C57BL/10 GnDg x SJL/J) F₁	16 - 17	-	N	-	2.5	*in vitro*	8 – 14	-

Model: Synchronous ET, E3.5/R3.5

McLaren & Michie, 1955	11	15	C3H x C57BL	17.5	prepub	SO	U	3.5	*in vivo*	mean of 7	-
Paria, et al., 1993	47	5	CD-1	4.5	adult	N	-	3.5	*in vivo*	12	5 - 7
Fossum, et al., 1989	50	4	C6B3F1	8.5	adult	N	-	3.5	*in vitro*	5	5
Paria, et al., 1993	51	5	CD-1	4.5	adult	N	-	3.5	*in vivo*	12	5 - 7
Wakuda, et al., 1999	51	20	ICR	11	adult	SO	U	1.5	*in vivo*	6	3
Paria, et al., 1993	52	5	CD-1	4.5	adult	N	-	3.5	*in vivo*	12	5 - 7
Song, et al., 2007	52	5	-	5.5	adult	N	-	3.5	*in vivo*	12	-
Goto, et al., 1993	58	16	ICR	13.5	adult	SO	U	3.5	*in vivo*	max 7	-
Rulicke et al., 2006	79	2	Zbz:FM	18	adult	SO	U	1.5	*in vitro*	7	7

Model: Asynchronous ET, E3.5/R4.5

Wakuda, et al., 1999	3	20	ICR	11	adult	SO	U	3.5	*in vivo*	6	3
Rulicke, et al., 2006	36	3	Zbz:FM	18	adult	SO	U	1.5	*in vitro*	mean of 8	mean of 8
Song, et al., 2007	48	7	-	6.5	adult	N	-	3.5	*in vivo*	12	-

Model: Asynchronous ET, E3.5/R5.5

Rulicke, et al., 2006	0	3	Zbz:FM	18	adult	SO	U	1.5	*in vitro*	mean of 7	mean of 7
Wakuda, et al., 1999	0	20	ICR	11	adult	SO	U	3.5	*in vitro*	6	3
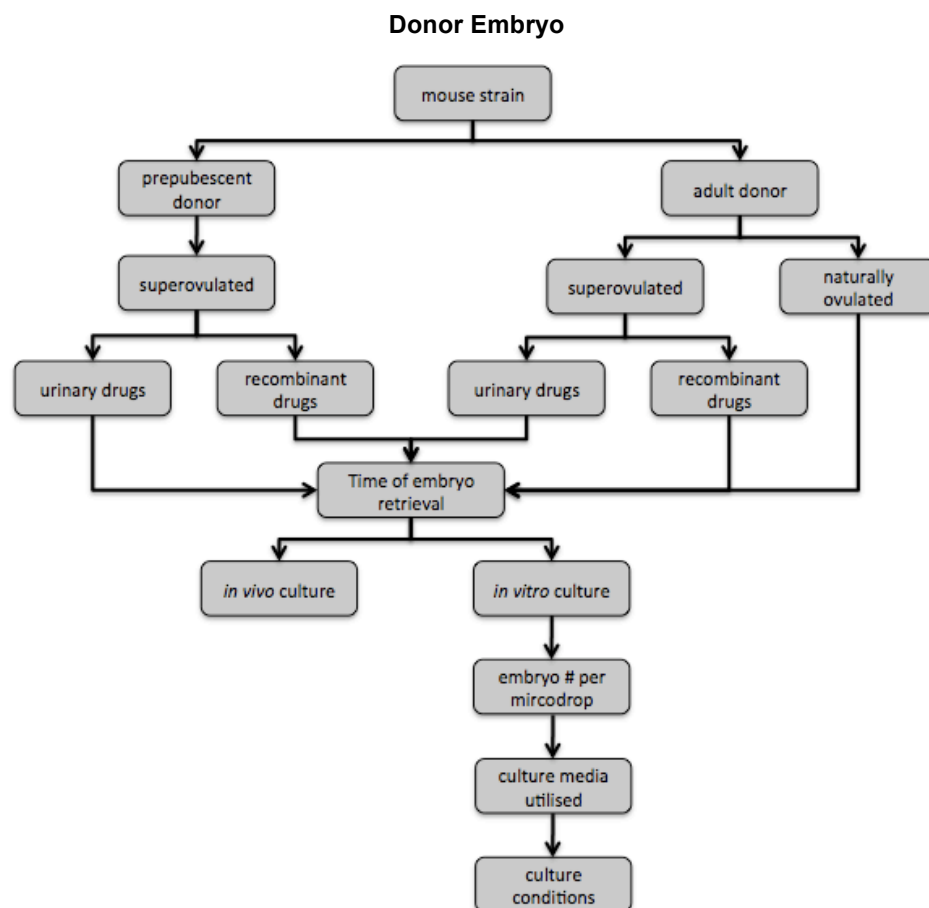
Donor Embryo

Figure 4 Examples of donor embryo variables illustrated by flow diagram. If the time of embryo retrieval was excluded, at least 10 possible scenarios result.
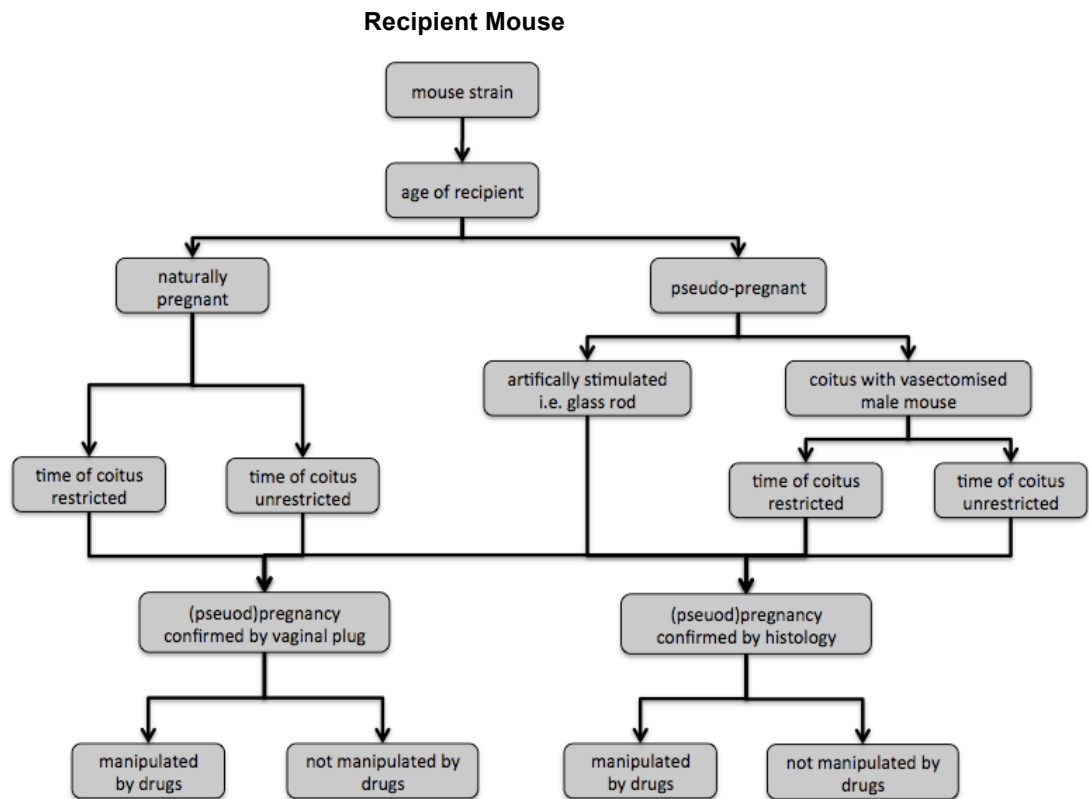
Recipient Mouse

Figure 5 Examples of recipient mouse variables illustrated by flow diagram. Twenty possible scenarios result from this flowchart.

Embryo Transfer Surgery
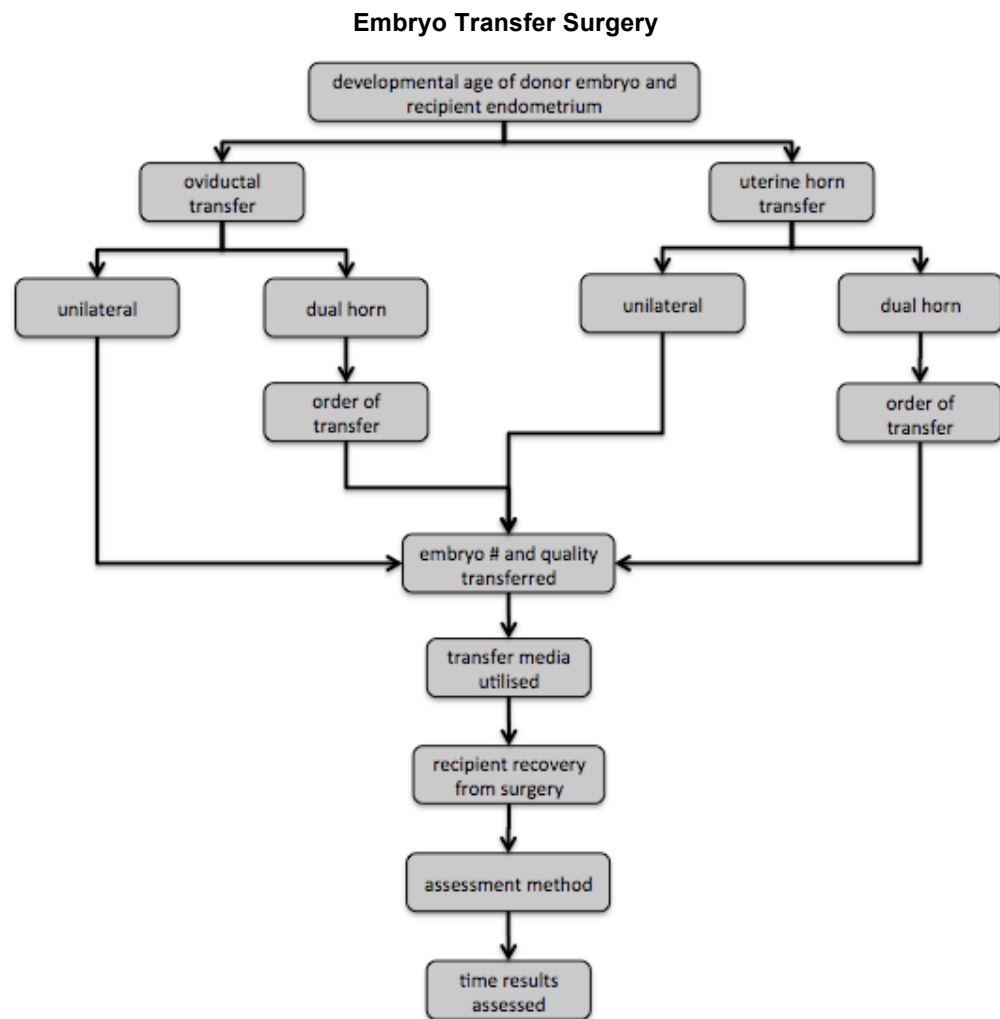
Figure 6 Examples of ET surgery variables illustrated by flow diagram. A minimum of four scenarios result.

2.2.3.3. The beginning of murine endometrial receptivity has not been clearly defined

A lack of clarity surrounded the beginning of murine endometrial receptivity, which is also known as the "window of implantation" (Edwards & Steptoe, 1983; Finn & McLaren, 1967; Paria, et al., 1993; Potts, 1968; Song, et al., 2007; Ueda, et al., 2003; Wakuda, et al., 1999). As the terminology suggests, implantation is only allowed for a finite period of time. Therefore, the detection of implants provided indirect evidence of endometrial receptivity (Psychoyos, 1961, 1973a, 1973b). Of the six studies identified, only two could suggest the beginning of murine endometrial receptivity within natural pregnancy (Finn & McLaren, 1967; Potts, 1968). The key difference lay in their experimental design.

Paria et al. (1993), Song et al. (2007), and Wakuda et al. (1999) assessed for the presence of implants no earlier than day-4.5 pc, located after the completion of endometrial receptivity. However, to effectively detect the beginning of that receptivity, mice must be assessed for the presence of implants both before and during that period of time. Nevertheless, the experimental design utilised by Paria et al. (1993), Song et al. (2007), and Wakuda et al. (1999) could have indicated an extension of endometrial receptivity. Together, the studies transferred E3.5 embryos into R0.5 through to R5.5 pseudo-pregnant recipient mice. Such ET spanned the pre-receptive, receptive and refractive endometrial phases. The detection of implants within recipient mice, whose endometrium was thought to be refractive at the time of ET, could have suggested extended endometrial receptivity (Paria, et al., 1993; Song, et al., 2007; Wakuda, et al., 1999).

In contrast, Finn and McLaren (1967), and Potts (1968) suggested the beginning of endometrial receptivity within natural pregnancy, albeit through a limited number of time-points. Finn and McLaren provided the earliest indication of murine endometrial receptivity at 93 hours pc (1967). However, neither their study nor any other, reported clear data earlier than 93 hours pc. As a result, published data was not inclusive of both the pre-receptive and receptive murine endometrial phases. For this reason, Finn and McLaren could only indicate that naturally pregnant mouse endometrium was receptive by 93 hours pc (1967).

Interestingly, McMaster, Dey and Andrews (1993) detected implantation, and thus endometrial receptivity one hour (92 hours pc) earlier than Finn and McLaren (1967). It was plausible that the natural and subtle variability found between mice, accounted for the one-hour difference. However, such an interpretation was difficult to validate as Finn and McLaren assessed only one naturally pregnant mouse at each time-point (1967). Thus, the extent of mouse-to-mouse variability within their work could not be determined. Additionally, the purpose of the McMaster et al. (1993) was to examine the distribution and activation of monocytes and neutrophils within murine uteri, which included implantation sites. Consequently, McMaster et al. (1993) simply referred to the time implantation was detected, which inadvertently was the earliest report within the literature.

The work of Ueda et al. (2003) is also worthy of noting. In contrast to Finn and McLaren (1967), and Potts (1968), Ueda et al. (2003) produced donor embryos by IVF, and utilised oviductal ET into pseudo-pregnant recipient mice. The asynchronous

oviductal transfer of E2.5 embryos into R0.5 recipient mice demonstrated endometrial receptivity by 50 hours pc. However, the inclusion of IVF and asynchronous oviductal ET made findings difficult to translate to natural mouse pregnancy. In contrast, the synchronous oviductal transfer of E0.5 embryos into R0.5 recipient mice, which was more analogous to the natural scenario, demonstrated endometrial receptivity by 98 hours pc. Twenty-four hours earlier zero implantation was detected. Thus, it was likely that endometrial receptivity began between 74 and 98 hours pc. Nonetheless, the well-designed research of Ueda et al. (2003), clearly served their overall objective, which was to investigate the embryo's ability to expand endometrial receptivity following oviductal ET. Section 2.2.3.4 would expand upon these results.

2.2.3.4. Impact of altered embryo-endometrial developmental synchrony on the timing of endometrial receptivity requires further investigation

Historically, endometrial development is considered independent of the developing pre-implantation embryo (Dickmann & Noyes, 1960; Doyle, et al., 1963; Psychoyos, 1973). For this reason, the timing of endometrial receptivity is thought to be unaffected by a shift in normal embryo-endometrial developmental synchrony as a product of ET. The pioneering work of Dickmann and Noyes (1960), and Doyle et al. (1963) helped establish this foundational knowledge. Their findings indicated that rat and murine embryos, whose development was more mature than their corresponding recipient endometrium, implanted at a comparable time to those synchronously developed embryos following ET. In fact, data suggested that pre-implantation embryos held the ability to pause in their development and wait for the developing endometrium. The embryonic ability to enter a dormant state, known as diapause, is well documented in up to 100 different species (Renfree, 1982; Renfree & Shaw, 2000). Some of which include: marsupials, seals, and rodents. This phenomenon, which can be driven by seasonal changes or lactation, can also be induced artificially within ovarectomised recipient mice, which have had continued exogenous hormonal support (Gidley-Baird, 1981; Paria, et al., 1993; Psychoyos, 1973; Renfree, 1982; Renfree & Shaw, 2000). Thus, the developmental maturity of pre-implantation embryos appears to hold little influence over the timing of endometrial receptivity.

Contrary to Dickmann and Noyes (1960), and Doyle et al. (1963), a limited number of murine studies have revealed the pre-implantation embryo's potential ability to influence endometrial development and thus the timing of receptivity (Shiotani, et al., 1993; Ueda, et al., 2003; Wakuda, et al., 1999). The *in vitro* murine work of Shiotani

et al. (1993) demonstrated significantly improved levels of donor embryo attachment to naturally pregnant explants, which had been exposed to naïve embryos prior to dissection. In addition, the *in vivo* work of Ueda et al. (2003), and Wakuda et al. (1999) was able to demonstrate the pre-implantation embryo's ability to bring forward the beginning and extend the later stages of endometrial receptivity. Furthermore, the influence of embryo-endometrial cross-talk on receptivity has been demonstrated in species outside the mouse, for example, bovine and horse (Mansouri-Attia, et al., 2009; Van Soom, Wydooghe, Heras, & Vandaele, 2011). As this research focused on *in vivo* implantation murine models that were established by a shift in embryo-endometrial developmental synchrony, a special interest was taken in the findings of Ueda et al. (2003) and Wakuda et al. (1999).

Ueda et al. (2003) reported that pre-implantation embryos were able to induce early endometrial receptivity. Both E0.5 and E2.5 embryos were transferred into the oviducts of R0.5 recipient mice (one developmental age per oviduct). Assessment of recipient mice revealed that the majority of E2.5 embryos had implanted at least one day earlier (day-3.5 pc) than their synchronous E0.5 counterparts (day-4.5 pc; Figure 7). In fact, implantation of E2.5 embryos was observed as early as day-2.5 pc. Although implantation frequencies of each group of embryos were not significantly different on this day, implants resulting from E0.5 embryos were not observed until day-4.5 pc.

Detecting implants as early as day-2.5 pc was unexpected given that the endometrium would normally be considered pre-receptive, presenting a natural barrier to implantation up to late day-3.5 pc (Carson, et al., 2000; Finn & McLaren, 1967; Ueda, et al., 2003). However, the majority of transferred E2.5 embryos would have become implantation-competent (E4.5) by day-2.5 pc (Ueda, et al., 2003). In contrast, the synchronously developed E0.5 embryos required at least another 1.5 days of development to become implantation-competent (Ueda, et al., 2003). Given that endometrial receptivity is thought to be at least 12 hours, but less than 24 hours in length, the findings of Ueda et al. (2003) suggested that pre-implantation embryos may be able to both bring forward and expand endometrial receptivity (Doyle, et al. 1963; Ma, et al., 2003; Paria, et al., 1993; Psychoyos, 1973a, 1973b; Yoshinaga, 1988).

Wakuda et al. (1999) suggested that the oviductal presence of pre-implantation embryos had the ability to extend murine endometrial receptivity. Prior to ET, pregnant and pseudo-pregnant recipient mice underwent oviductal ligation (day-0.5 pc). As a

result, naïve pre-implantation embryos were held within the oviduct of pregnant recipient mice, whereas the oviducts of pseudo-pregnant recipient mice were void of pre-implantation embryos (Wakuda, et al., 1999). Within recipient mice whose oviducts contained naïve pre-implantation embryos, ET of E3.5 donor embryos performed up to day-5.5 pc successfully resulted in implants (Figure 8). Not only was this one day later than their pseudo-pregnant counterparts (day-4.5 pc), but murine endometrium would be normally considered refractive by day-5.5 pc (Hogan, et al., 1994; Wakuda, et al., 1999).
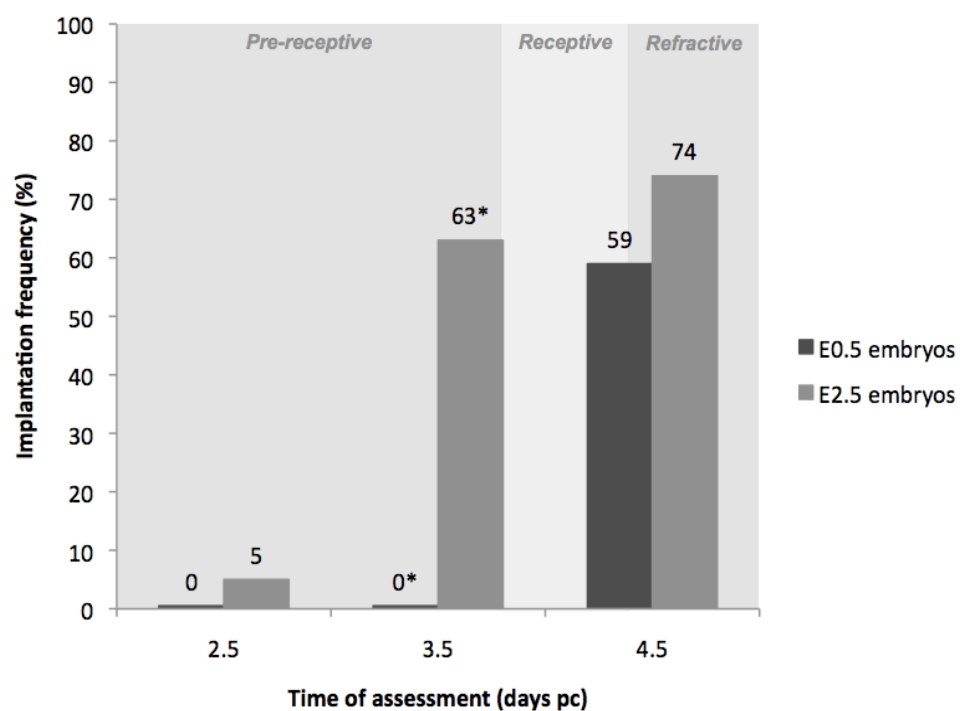
Figure 7 Implantation frequencies detected by Ueda et al. (2003) following the transfer of E0.5 and E2.5 embryos into 81 R0.5 recipient mice. Implantation frequencies resulting from E0.5 and E2.5 embryos significantly different on day-3.5 pc (*, $p < 0.05$).
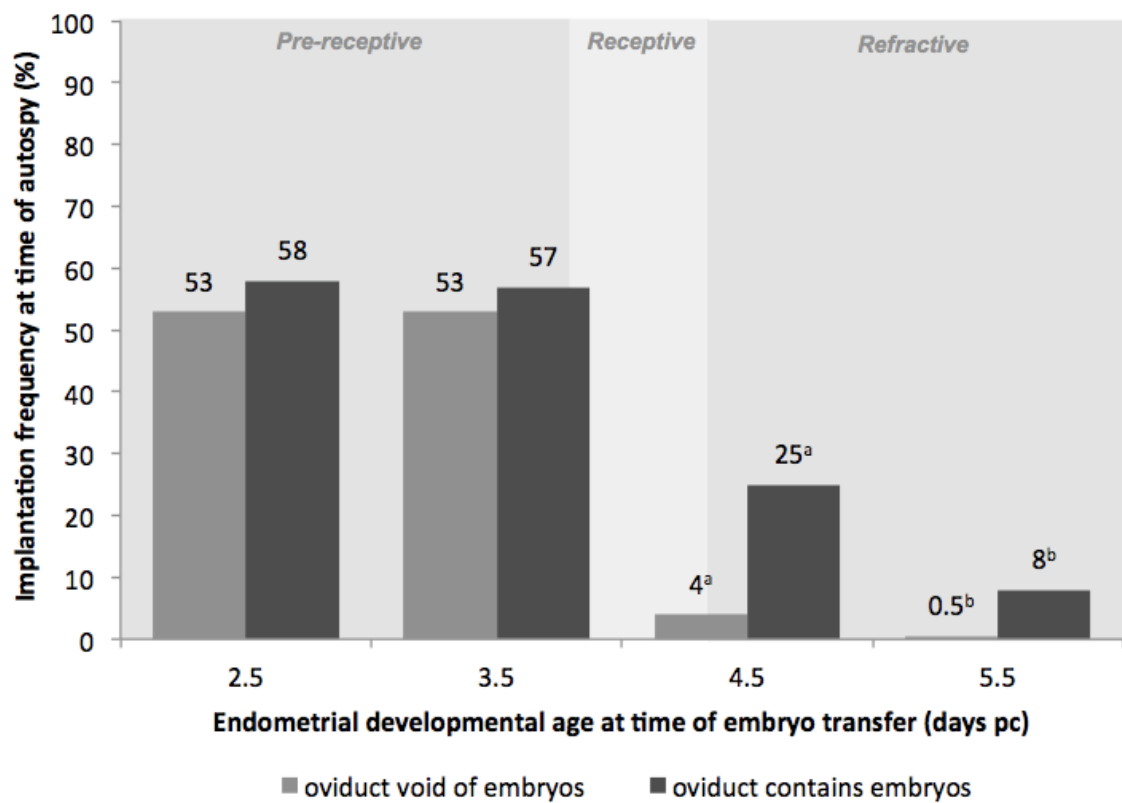
Figure 8 Implantation frequencies detected by Wakuda et al. (1999) following ET into recipient mice whose oviducts contained or were void of naïve pre-implantation embryos. A total of 128 recipient mice were used. Autopsies performed after endometrial receptivity (day-11 pc). Shaded areas indicated the expected endometrial developmental state at the time of ET. Values marked with a or b indicate significant difference calculated by Wakuda et al. (1999) ($p < 0.05$).

Although the work of Dickmann and Noyes (1960), and Doyle et al. (1963) was ground-breaking, coming at a time where endometrial receptivity knowledge was still in its infancy, these pioneers did not have access to the expansive knowledge, skill base, money, nor equipment that present day researchers now enjoy. Therefore, looking through the advanced glasses of present day knowledge, it is possible that the experimental design utilised by these researchers may not have been sensitive enough to pick up any differences. For example, while the detection of implants remains a simple, yet effective methodology to indicate the timing of endometrial receptivity, its level of efficiency is dependent on two variables (Psychoyos, 1961, 1973a, 1973b). These include: 1) the technique used to detect the implants; and 2) the time and frequency of implantation assessment. Evaluating the experimental design utilised by Dickmann and Noyes (1960), and Doyle et al. (1963), in context of these two variables, revealed a number of factors, which could account for the potential lack of sensitivity.

The intravenous administration of blue dye prior to autopsy is well documented as an effective technique to detect increased vascularisation surrounding the implantation site, the first gross indication of implantation (Psychoyos, 1961). However, Dickmann and Noyes (1960), and Doyle et al. (1963) utilised alternative methodology, which was likely due to the slow dissemination of knowledge at that time. Their methodology included: a) percentage frequencies of recovered embryos subsequent to transfer; and b) gross morphological identification of implantation sites through light microscopy. Both Dickmann and Noyes (1960), and Doyle et al. (1963) appeared to negatively correlate implantation frequency with the percentage frequency of recovered embryos. However, there was no way to distinguish between unrecovered embryos that were too firmly attached and those that had been lost pre-implantation. As a result, there was the risk that implantation frequencies were masked and could be much lower than the percentage frequency of recovered embryos indicated. The second alternative methodology also had its own set of limitations. The gross morphological identification of implantation sites through light microscopy relied on sufficient lighting and could therefore be prone to subjectivity (Dickmann & Noyes, 1960; Doyle, et al., 1963).

In context of the time and frequency of implantation assessment, the second variable to consider, it is important that assessed time-points span both the pre-receptive and receptive endometrial phases. Time-points should also be frequent enough so that vital data are not omitted. The earlier work of Dickmann and Noyes (1960) assessed recipient rats at only two different time-points, which were 24 hours apart (days-4.5

and 5.5 pc). Although time-points spanned both the pre-receptive and receptive rat endometrial phases, an interval of 24 hours could have missed subtle differences. This potential risk was highlighted through the dramatic reduction in mean numbers of recovered rat embryos within natural pregnancy over a period of 12 hours (Dickmann & Noyes, 1960). On 5.0-days pc of natural rat pregnancy, a mean of 3.3 embryos were recovered. Twelve hours later (day-5.5 pc), a mean of only 0.5 embryos were recovered. Consequently, a time interval of 24 hours was possibly too large to detect any subtle differences. In contrast, Doyle et al. (1963) assessed mice every one to two hours, from 80 hours pc (day-3.5 pc) through to 109 hours pc (day-4.5 pc). Therefore, time-points were more likely to capture both the pre-receptive and receptive endometrial phases, but were also frequent enough to capture more detailed information.

Three further variables that may have desensitized the experimental design utilised by Dickmann and Noyes (1960), and Doyle et al. (1963) included: 1) inconsistent or limited numbers of transferred embryos and recipient mice; 2) rat uteri anatomy; and 3) possible technician inconsistency. Inconsistent numbers of transferred embryos can lead to varied implantation probability (Lambers, et al., 2007; Matorras, et al., 2005). Mathematical modelling demonstrated that with every successful embryo implantation, the implantation probability of subsequent embryos increased (Matorras, et al., 2005; Ye, 2006). Utilizing inconsistent or limited numbers of recipients also made it difficult to determine technician consistency and mouse-to-mouse variability. A larger cohort of recipient mice, for each assessed time-point, would have accounted for any potential natural variation seen between recipient mice.

Rat uteri lack the partial septum between the left and right uterine horns, as observed within mice (Young, 1952 as cited in Rulicke, et al., 2006). As a consequence, transmigration, the movement of embryos from one uterine horn to the other, is possible with rats (Rulicke, et al., 2006). Within Dickmann and Noyes (1960), R3.5 recipient rats received E3.5 or E4.5 embryos, or both (separate uterine horns). Within R3.5 recipient rats that received both groups of embryos, E4.5 embryos could transmigrate into the opposite uterine horn and implant alongside E3.5 embryos. Numbers of implants that were thought to result from E3.5 rat embryos could be misleadingly comparable to E4.5 rat embryos. Therefore, transferring two different age groups of donor embryos into the same recipient was unsuitable within rats (Dickmann & Noyes, 1960).

The final variable to consider, which may have reduced experimental design sensitivity within Doyle et al. (1963), could have been technician inconsistency. Unlike Dickmann and Noyes (1960), percentage frequencies of recovered embryos did not decrease over time. Instead, both prominent peaks and extreme troughs were apparent (Figure 9). In addition to variable or limited numbers of recipient mice, inconsistent technique could have accounted for such data. For example, if the uterus of one recipient mouse were flushed more vigorously than another from a second recipient mouse, the delicate interactions associated with early implantation would be exposed to higher shear forces. As a consequence, implanted embryos could be dislodged, resulting in increased percentage frequencies of recovered embryos and thus misleadingly low implantation rates (Dickmann & Noyes, 1960).
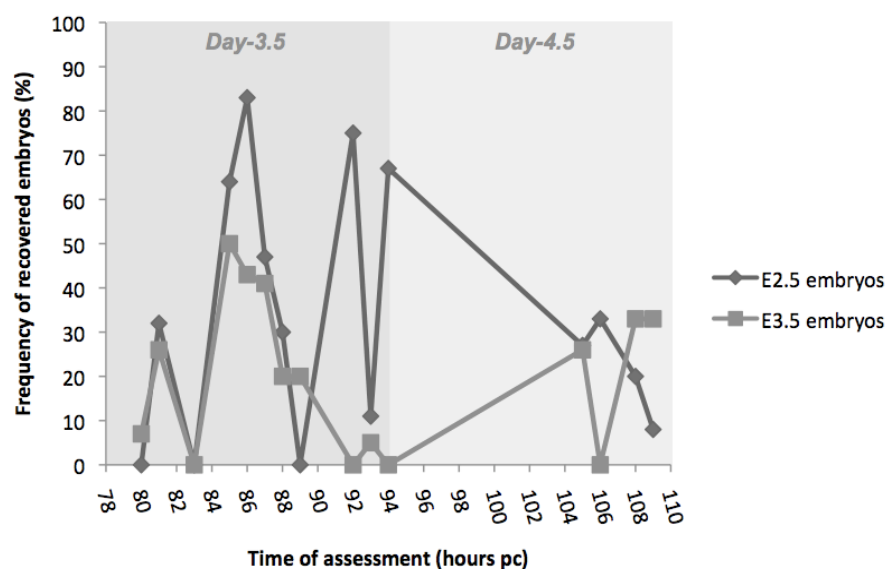
Figure 9 Percentage frequencies of embryos recovered by Doyle up to two days after the transfer of both E2.5 and E3.5 embryos into 44 R2.5 recipient mice. Percentage frequencies of recovered embryos were thought to be inversely proportional to percentage frequencies of implants (implantation rate). Data adapted from Doyle et al. (1963).

Present-day hindsight surrounding the findings of Dickmann and Noyes (1960) and Doyle et al. (1963) illuminated the need to revisit the pre-implantation embryo's ability to influence endometrial development. Although, preliminary studies surrounding this ability demonstrated substantial promise, gaps in knowledge still remained (Shiotani, et al., 1993; Ueda, et al., 2003; Wakuda, et al., 1999.) Current knowledge was unable to clearly reveal how the developmental maturity of pre-implantation embryos, and thus the resulting shift in normal embryo-endometrial developmental synchrony, could

influence the timing of endometrial receptivity following ET. Any potential shift, extension or expansion of endometrial receptivity could alter the opportunity to implant. Therefore, it was important to improve fundamental knowledge surrounding this subject, for you cannot effectively control what you do not understand.

2.2.3.5. Impact of altered embryo-endometrial developmental synchrony on implantation rates is unclear

Embryo-endometrial developmental synchrony following murine-ET is influenced by a number of different variables. However, for ease of explanation, these can be reduced to two broad variables. The first of these includes the length of time required by donor embryos to reach implantation-competency, which is dependent on embryo developmental age at the time of transfer. The second variable includes the length of time available for donor embryos to reach that competency and implant, which is dependent on recipient endometrial developmental age at the time of transfer relative to the completion of endometrial receptivity. If the length of time required and available for embryo implantation-competency is comparable, implantation can occur (Schematic A, Figure 10). Likewise, if the time available exceeds the time required by donor embryos, implantation can also occur (Schematic B, Figure 10). However, when the length of time required exceeds what is available for embryo implantation-competency, implantation cannot occur (Schematic C, Figure 10). Although implantation opportunity can be clearly linked with both the length of time required and available for embryo implantation-competency, their combined influence on implantation rates remains somewhat ambiguous.

Two studies, illustrated in Figure 11, demonstrated a negative correlation between the length of time required for embryo implantation-competency and implantation rates. McLaren and Michie (1995) performed a series of synchronous and asynchronous ET into naturally pregnant recipient mice. Subsequent to the transfer of E3.5 embryos into R2.5 recipient mice, 23% of transferred embryos resulted in implants. In contrast, only 11% of their E2.5 counterparts, which were also transferred into R2.5 recipient mice, implanted (McLaren & Michie, 1955). The transfer of E3.5 or E2.5 embryos into R3.5 recipient mice demonstrated a comparable pattern. Eleven percent of transferred E3.5 embryos successfully implanted, whereas zero E2.5 embryos, which required an additional 24 hours development, resulted in an implant (McLaren & Michie, 1955).
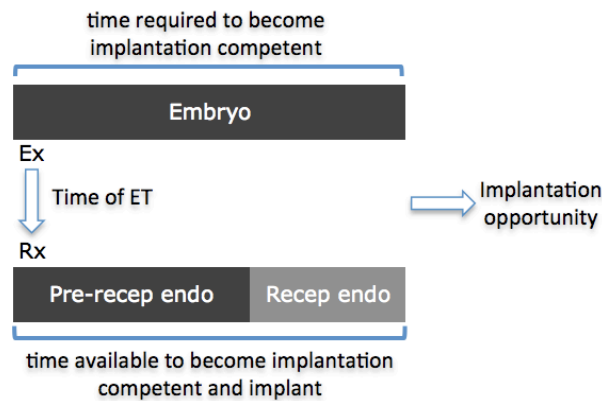
Schematic A - Time required to reach implantation-competency comparable to time available
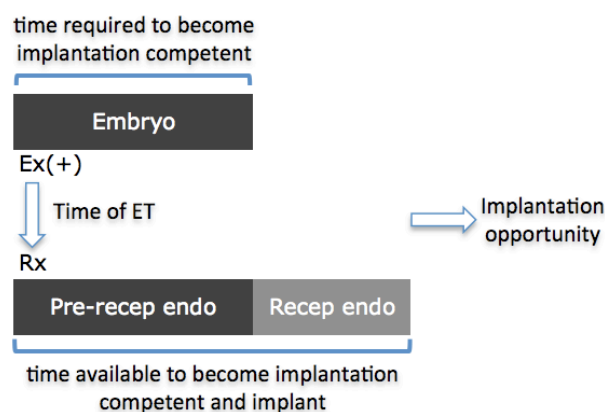
Schematic B - Time required to reach implantation-competency less than time available
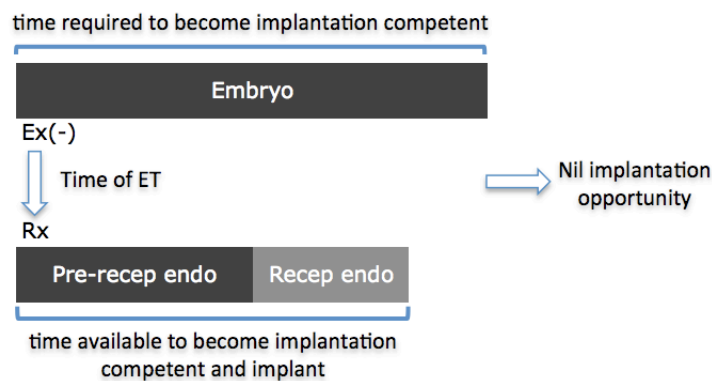
Schematic C - Time required to reach implantation-competency more than time available

Figure 10 Schematic illustrating the impact of the time required and available for embryo implantation-competency on implantation opportunity. Within Schematic A, the time required and available to reach embryo implantation-competency is comparable, leading to implantation opportunity. Within Schematic B, the time available exceeds what is required to reach embryo implantation-competency, also leading to implantation opportunity. Within Schematic C, the time required exceeds what is available to reach embryo implantation-competency, leading to no opportunity to implant. Key: Pre-receptive endometrium (pre-recep endo); receptive endometrium (recep endo); Ex, embryo developmental age x-number of days pc; Rx, recipient mouse endometrium developmental age x-number of days pc.

The low levels of implantation observed could have been due to number of reasons. Two examples included the to competitive implantation with naïve embryos or the developmental nature of ET at the time of publication. Again the innovative work of McLaren and Michie (1955), was not privy to the extensive knowledge, recognised techniques, money, nor specialised equipment available within today's scientific community. Despite the low levels of implantation observed by McLaren and Michie (1955), data indicated that as the time required by embryos to reach implantation-competency increased, resulting implantation rates decreased.

Goto et al. (1993) also demonstrated a negative correlation between the time required for embryo implantation-competency and implantation rates. As seen in Figure 11, 58% of E3.5 embryos resulted in implants following their transfer into R3.5 recipient mice. In contrast, zero E1.5 embryos resulted in an implant subsequent to their transfer into R3.5 recipient mice (Goto, et al., 1993). At the time of transfer, E1.5 embryos required at least three additional days to become implantation-competent (Armant, 2005). Such development translated to day-6.5 pc within the recipient mouse, whose endometrium would normally considered refractive by that time (Finn & Martin, 1974; Psychoyos, 1973a, 1973b). As a result, zero implantation subsequent to the transfer of E1.5 embryos was not considered unusual.

Despite the apparent negative correlation between the lengths of time required for embryo implantation-competency and implantation rates, the literature lacked clarity surrounding the influence of the time available for that competency. Graph A of Figure 12 demonstrates a positive correlation between the time available for embryo implantation-competency and implantation rates (Ertzeid & Storeng, 2001; Fossum, et al., 1989; Kelley, et al., 2006; McLaren & Michie, 1955; Paria, et al., 1993; Rulicke, et al., 2006). These six studies, which utilised synchronous and asynchronous embryo transfer, and ET into SO recipient mice, were able to indicate that as the time available for embryo implantation-competency increased, so did resulting implantation rates.

In contrast, a further five studies, illustrated in Graph B of Figure 12, demonstrated indistinct correlation (Goto, et al., 1993; Song, et al., 2007; Wakuda, et al., 1999). The collection of studies utilised synchronous and asynchronous ET, each resulting in a shift from normal embryo-endometrial developmental synchrony. Resulting implantation rates were either comparable or increased when the length of time available to donor embryos decreased. This lack of correlation was likely due to the numerous experimental variables between individual studies (Table 4). Two of which

included: 1) variable numbers of recipient mice, which could lead to differences in statistical strength; and 2) variable numbers of embryos transferred, which could lead to dissimilar implantation probabilities (Goto, et al., 1993; Lambers, et al., 2007; Matorras, et al., 2005; Song, et al., 2007; Wakuda, et al., 1999; Ye, 2006). Consequently, Graphs A and B (Figure 12) could not clearly elucidate how the time available for embryo implantation-competency influenced implantation rates.

McLaren and Michie (1955) published the sole study that could suggest how both the time required and available for embryo implantation-competency, and thus an alteration in embryo-endometrial developmental synchrony, could influence implantation rates. The remainder of studies often focussed on only one of the two variables, which led to incomplete picture within their chosen experimental design. Although McLaren and Michie (1955) provided a useful insight, the literature as a whole was unable to clearly elucidate how a shift in embryo-endometrial developmental synchrony could influence implantation rates. Thus, the importance of improved fundamental knowledge surrounding murine implantation rates, and the standardization of those *in vivo* implantation murine models available, has been highlighted. Gaining such knowledge would strengthen experimental data, allow for enhanced comparison between research groups and help establish research platforms, effective to explore implantation and IVF-interventions. For this reason, the overall objective of this research was to gain an understanding into improved standardization of *in vivo* implantation murine models, which were suitable to explore implantation and preliminary studies surrounding IVF-intervention potential.
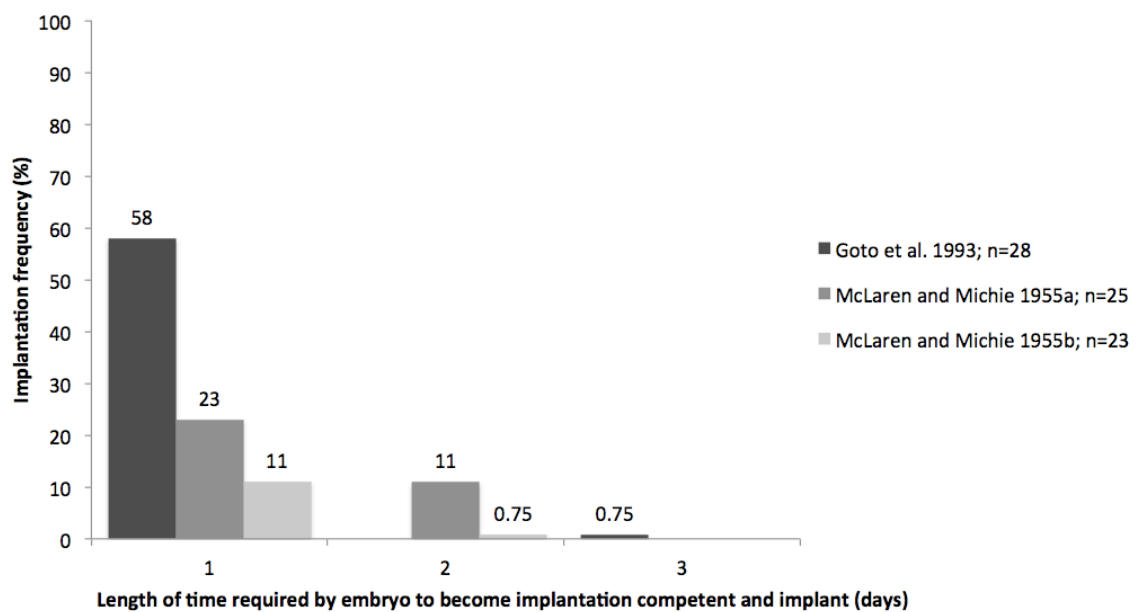
Figure 11 Impact of the time required for embryo implantation-competency on implantation rates found within the literature. Implantation rates were expressed as the percentage frequency of the total numbers of embryos transferred. Key: recipient number (n).
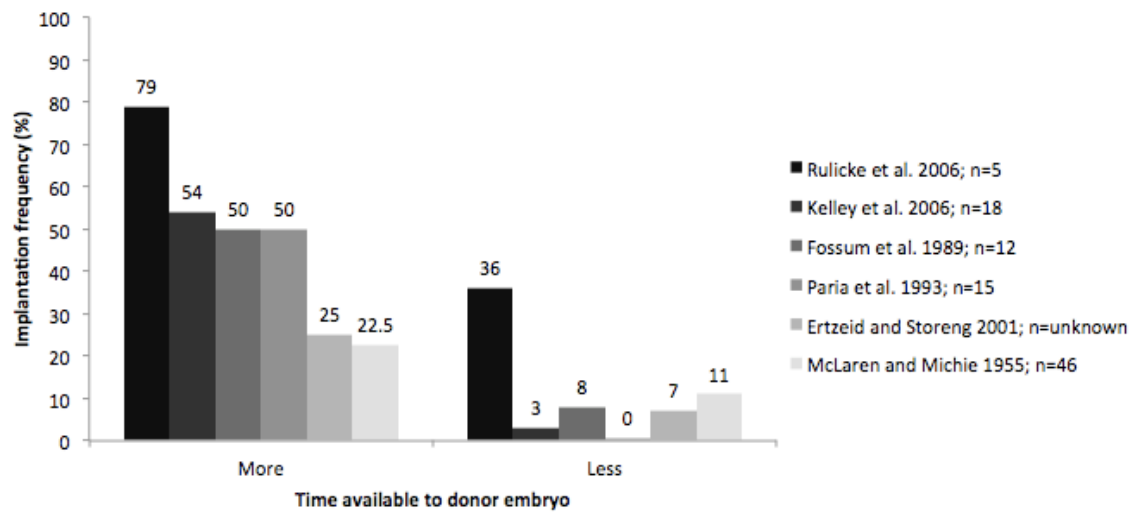
Graph A - Implantation rates decrease when less time is available for embryo implantation-competency
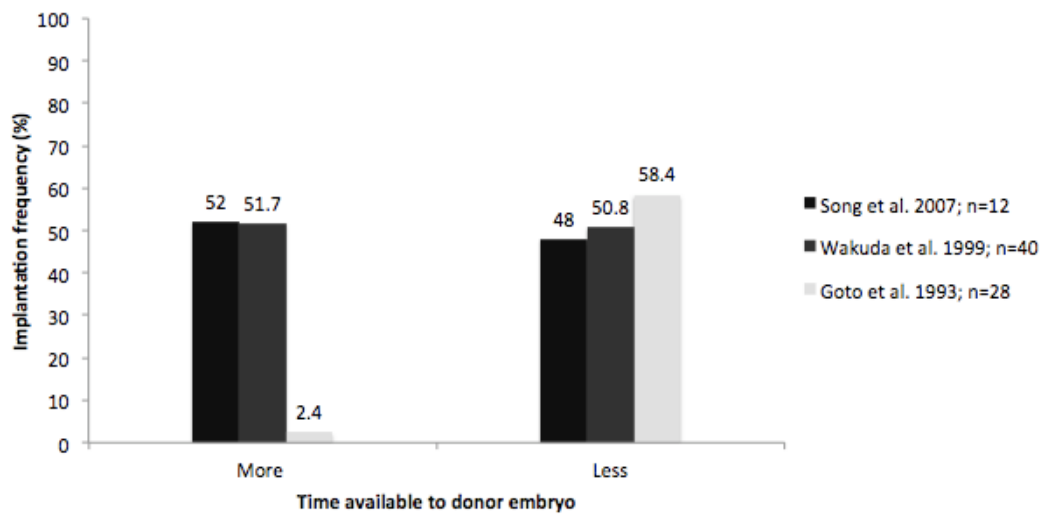
Graph B – No clear link between the time available for embryo implantation-competency and implantation rates

Figure 12 Impact of the time available for embryo implantation-competency on implantation rates found within the literature. Graph A includes studies that utilised synchronous and asynchronous ET, and ET into SO-recipient mice. Graph B includes studies that utilised synchronous and asynchronous ET. Key: recipient number (n).

Table 4 Experimental variables associated with the studies illustrated in Graph B, Figure 12. Key: Syn/Asyn, synchronous and asynchronous ET; SO, superovulated; N, naturally ovulated; and U, urinary.

Researcher	Model type	Recipient mouse strain	Recipient number	Time of assessment (days pc)	Embryo donor mouse age	Naturally or superovulated	Urinary or recombinant gonadotropin	Day of embryo retrieval (pc)	Embryo culture status	Number of embryos transferred per recipient	Number of embryos transferred per uterine horn	Unilateral or dual horn transfer
Goto 1993	Syn/Asyn	ICR	12	11.5	adult	SO	U	3.5	*in vivo*	5 - 7	-	Unilateral
Wakuda et al., 1999	Syn/Asyn	ICR	20	11	adult	SO	U	3.5	*in vivo*	6	3	Dual horn
Song et al., 2007	Syn/Asyn	-	5	5.5	adult	N	-	3.5	*in vivo*	12	-	Dual horn

3   Materials and methodology

3.1 *Materials*

3.1.1   Laboratory equipment

As seen in Table 5, items are organised into the following categories: consumables; reagent preparation; surgical equipment; and large equipment. Within each category, items are organized alphabetically.

Table 5 Laboratory equipment details. Items were categorised into consumables, reagent preparation, surgical equipment and large equipment.

Item	Catalogue Number	Manufacturer details
Consumables		
Blutac	-	Bostick 211 Boston Street, Middleton, MA 01949, United States of America
Capillary tubes, 75mm	41A2502	Kimble-Chase, 1022 Spruce Street, Vineland, NJ 08362-1502, United States of America
Centrifugal units, 0.2 µm filter, 500 µL total volume, sterile	UFC30GV0S	© EMD Millipore Corporation, 290 Concord Road, Billerica, MA 01821, United States of America
Conical tubes, 10 mL, sterile	188271	Greiner Bio-one GmbH, Maybachstrasse 2, 72636, Frickenhausen, Germany
Conical tubes, 50 mL, sterile	GR227261	Greiner Bio-one GmbH, Maybachstrasse 2, 72636, Frickenhausen, Germany

Culture dish, 35 x 10 mL, vented, polystyrene, sterile	627160	Greiner Bio-one GmbH, Maybachstrasse 2, 72636, Frickenhausen, Germany
Face mask	-	Health Support Ltd, PO Box 44-027 Pt Chevalier Auckland 1246, New Zealand
Filter units, Steritop-GP, 0.22 µm, polyethersulfone, 500 mL 45 mm, radio-Sterilised	SCGPT05RE	© EMD Millipore Corporation, 290 Concord Road, Billerica, MA 01821, United States of America
Filter unit, syringe driven Millex – GVO 0.25 µm pore, 33 mm membrane diameter, sterile	SLGV033RS	© EMD Millipore Corporation, 290 Concord Road, Billerica, MA 01821, United States of America
Hairnets	-	Health Support Ltd, PO Box 44-027 Pt Chevalier Auckland 1246, New Zealand
Insulin syringes with 29 G needle, 1 mL, sterile	SYR121	Becton, Dickinson and Company©, 8 Pacific Rise, Mt Wellington, Panmure, Auckland 1741, New Zealand
IVF culture plate, 4-well, sterile	NUN1444444	Nunc, Kamstrupvej 90, Post box 280, DK-4000, Roskilde, Denmark
Microcentrifuge tubes, 1.6 mL, polypropylene	3745.S.X	Neptune, 9880 Mesa Rim Road, San Diego, CA 92121, United States of America
Paper towels	Interfold towels	Hygenex Corp. 4034 Appleton Ave, Roanoke, VA 24042, United States of America
PCR tubes, 0.6 mL, flat-cap, sterile, RNase, DNase, DNA and pyrogen free	3737.S.X	Neptune, 9880 Mesa Rim Road, San Diego, CA 92121, United States of America
Petri dish, 90 x 100 mm, polystyrene, sterile	S9014-05	Techno Plas Pty. 8 Benjamin Street, St Marys, South Australia 5042
Pipette tips, 10 µL, sterile	2342.S	Neptune, 9880 Mesa Rim Road, San Diego, CA 92121, United States of America

Pipette tips, 200 µL, sterile	2102.NS	Neptune, 9880 Mesa Rim Road, San Diego, CA 92121, United States of America
Pipette tips, 1000 µL, sterile	2162.S	Neptune, 9880 Mesa Rim Road, San Diego, CA 92121, United States of America
Plastic pipettes, 1 mL sterile	604181	Greiner Bio-one GmbH, Maybachstrasse 2, 72636, Frickenhausen, Germany
Surgical suture, silk, HR 17 (1.5 G) half circle cutting needle, sterile	4027	Resorba Wundversorgung GmbH & Co. KG, Am Flachmoor 16, 90475 Nuremberg, Germany
Microscope slides for vaginal smears	1324	Global Scientific Inc., 610 Winters Avenue Paramus, NY 07652, United States of America
Mixed propane/butane gas cylinder	-	Purchased from any camping store
Overshoes	PCL090	Medex, P.O. Box 21, Kensington, MD, United States of America
Gloves, vinyl, powder free	ANSE8511	Ansell Ltd (Asia Pacific), 33 Wang Chiu Road, Kowloon Bay, 2610B-12A, 26/F., Exchange Tower, Kowloon 8523, Hong Kong S.A.R., China
Reagent Preparation		
Digital scales, accuracy 0.0001 g	PB102-S	Metler-Toledo Ltd., 220 Turner Street, PO Box 173, Port Melbourne, VIC 3207, Australia
Glass beakers, 10 – 1000 mL	-	Schott, Hattenbergstrasse 1055122 Mainz, Germany
Laminar Flowhood	NV-201-403E	Nuaire, 2100 Fernbrook Lane, Plymouth, MN 55447, United States of America
Microcentrifuge	-	Denver Instrument Company Ltd, 5 Orville Drive, Bohemia, NY 11716, United States of America

pH meter	Seven easy	Metler-Toledo Ltd., 220 Turner Street, PO Box 173, Port Melbourne, VIC 3207, Australia
P10 Eppendorf Pipettor	Eppendorf Research®	Eppendorf South Pacific Pty. Ltd. Unit 4 112 Talavera Road, North Ryde NSW 2113 Australia
P100 Eppendorf Pipettor	Eppendorf Research®	Eppendorf South Pacific Pty. Ltd. Unit 4 112 Talavera Road, North Ryde NSW 2113 Australia
P200 Eppendorf Pipettor	Eppendorf Research®	Eppendorf South Pacific Pty. Ltd. Unit 4 112 Talavera Road, North Ryde NSW 2113 Australia
P1000 Eppendorf Pipettor	Eppendorf Research®	Eppendorf South Pacific Pty. Ltd. Unit 4 112 Talavera Road, North Ryde NSW 2113 Australia
Shaking waterbath	Trecator 1024	-
Vacuum pump, Buchi	V500	BUCHI Labortechnik AG Meierseggstrasse 40 Postfach CH-9230 Flawil 1 Switzerland
Surgical equipment		
Binocular dissection microscope	Stemi SV 6	Carl Zeiss NZ Ltd, Service Medical Solutions,15B Paramount Drive, P.O. Box 12-1001, Auckland 1231, New Zealand
Bunsen burner, touchmatic	002807-000	Hanau, address not found
Digital camera, 4.2 Mega Pixels	Sony cyber shot	Sony New Zealand, Akoranga Business Park The Warehouse Way, Northcote 0626
Forceps, rats tooth, 11.5 cm	G062-061	Incus Surgical Ltd, Mabrook House, Bocking End, Braintree, Essex, CM7 9AA, England
Jewellers forceps, extra-fine precision, 11 cm	DIPFBSS5	A. DUMONT & Fils, Rue Théodore Dumont 1 – 2924 Montignez – Switzerland

Light source, cool	KL200	Carl Zeiss NZ Ltd, Service Medical Solutions,15B Paramount Drive, P.O. Box 12-1001, Auckland 1231, New Zealand
Moustache clippers	9985-612	Wahl Corporate, 2900 North Locust Street, Sterling, IL 61081, United States of America
Surgical scissors, fine, 11.5 cm	AC450/11NOPA	Nopa® Instruments, Medizintechnik GmbH. Weilatten 7-9 78532, Tuttlingen, Germany
Serrafine clamp, 3.8 cm	RS7440	Roboz Surgical Instrument Co., Inc. P.O. Box 10710 Gaithersburg, MD 20898-0710, United States of America
Thermo plate	MATS-U55S	Tokai hit Co. Ltd., 306-1, Gendoji-cho, Fujinomiya-shi, Shizuoka-ken, 418-0074, Japan

Large equipment

Biohazard safety cabinet, ESCO Airstream Class II	LA2-4A1	Esco Micro Pte Ltd, 21 Changi South Street 1, Singapore 486777
Cage bases, large, rat, dimensions 41 x 11 x 24 cm	-	Able Scientific, PO. Box 357, West Ryde 2114, New South Wales, Australia
Cage lids, high-top, rat, dimensions 42 x 9 x 25 cm	-	Able Scientific, PO. Box 357, West Ryde 2114, New South Wales, Australia
Cage lids, modified in-house high-top for restricted breeding, dimensions of holding cage 9 x 18 x 14, dimensions of main cage area 19 x 24 (surface area only)	-	Originals purchased from Able Scientific, PO. Box 357, West Ryde 2114, New South Wales, Australia
Cage bases, large, rat, dimensions 39 x 15 x 22 cm	-	University of Auckland, Private Bag 92019, Auckland 1142 New Zealand

Cage lids, flat, rat, dimensions 38 x 21 cm	-	University of Auckland, Private Bag 92019, Auckland 1142 New Zealand
Cage bases, small, mouse, dimensions 28 x 11 x 15 cm	-	University of Auckland, Private Bag 92019, Auckland 1142 New Zealand
Cage lids, flat, mouse, dimensions 27 x 14 cm	-	University of Auckland, Private Bag 92019, Auckland 1142 New Zealand
Gas flow regulator (for CO_2 euthanasia of mice)	105207	BOC, 988 Great South Road, Penrose, Auckland 1061, New Zealand
Gas regulator (for CO_2 incubators)	Baseline, dual mode gas regulator	BOC, 988 Great South Road, Penrose, Auckland 1061, New Zealand
Sanyo Incubator	MCO-20A1C	Panasonic Health Care Company of North America, 1300 Michael Drive, Suite A, Wood Dale, IL 60191, United States of America
Ultralow (-85°C) chest freezer	-	Nuaire, 2100 Fernbrook Lane, Plymouth, MN 55447, United States of America
Water bottles, with stoppers, 500 mL	-	Able Scientific, PO. Box 357, West Ryde 2114, New South Wales, Australia
Water bottles, with stoppers, 250 mL	-	University of Auckland, Private Bag 92019, Auckland 1142 New Zealand

3.1.2 Reagents

Stock reagent details are listed in Table 6. Preparation details of experimental reagents follow Table 6.

Table 6 Stock reagent details. Reagents are organized alphabetically and include a product description, storage specifications, catalogue numbers, and manufacturer details.

Product description	Storage specifications	Catalogue Number	Manufacturer details
Biotinylated ganglioside (BioG)	-80°C	-	Made in-house
Bovine albumin, fatty acid free, lypholized	4 – 5 °C	ABIVP-001	ICP Biotechnology – now Liquidated.
Calcium Chloride ($CaCl_2$), anhydrous powder, MW=110.99	RT	1.02378.0500	Merck New Zealand, 22 Hobill Avenue, Auckland 2104, New Zealand
CO_2 (small and large cylinders)	RT	-	Air Liquide New Zealand, 19 Maurice Ave, Penrose 1061, Auckland, New Zealand
D-Glucose ($C_6H_{12}O_6$), MW=180.16	RT	121341	Panreac, C/ Garraf 2, Polìgono Pla de la Bruguera, E-08211 Castellar del Vallès, Spain
DL-Lactic Acid, sodium salt, MW=112.1	4 – 5 °C	L7900	Sigma-Aldrich New Zealand Ltd., P.O. Box 106-406 Auckland, 1030 New Zealand

Dried synthetic glycolipid, FSL-A(GALNa3[Fa2]GALb)-SA1-L1 (FSL-A), functional head group is derived from blood group A trisaccharide GalNAcα3(Fucα2)Galβ, Molecular weight 1462.71 Da	-20°C	421604-1-R&D	KODE Biotech Ltd., PO Box 5965, Wellesley Street Auckland 1141, New Zealand
Dried synthetic glycolipid, FSL-Le^{Y}	-20°C	Discontinued	KODE Biotech Ltd., PO Box 5965, Wellesley Street Auckland 1141, New Zealand
Dried synthetic glycolipid FSL-HA$_{high}$, 20 – 100 saccharides, approximately molecular weight of 17 kDa	-20°C	Discontinued	KODE Biotech Ltd., PO Box 5965, Wellesley Street Auckland 1141, New Zealand
Dried synthetic glycolipid FSL-HA$_{mid}$, 30 - 40 saccharides, approximate Molecular Weight of 8 kDa	-20°C	Discontinued	KODE Biotech Ltd., PO Box 5965, Wellesley Street Auckland 1141, New Zealand
Extran® MA 03, phosphate free cleaning solution	RT	107550	Merck Millipore Headquarters, 290 Concord Road, Billerica, MA 01821, United States of America
High molecular weight Hyaluronan (HMW-HA), undigested, ≤ 1% protein. Soluble in aqueous solution at 5 mg/mL	-20°C	53747	Sigma-Aldrich New Zealand Ltd., P.O. Box 106-406 Auckland, 1030 New Zealand

Embryo culture media, BlastAssist®, for culture from the 4 – 8 cell stage	2 – 8°C, protect from light	12160010	Origio Medicult Media, Knardrupvej 2, 2760 Mâlov, Denmark
Embryo culture media, EmbryoAssist™, for culture from fertilization until 2 – 8 cell stage	2 – 8°C, protect from light	12130010	Origio Medicult Media, Knardrupvej 2, 2760 Mâlov, Denmark
Embryo transfer media containing hyaluronan, UTM™	2 – 8°C, protect from light	11520010	Origio Medicult Media, Knardrupvej 2, 2760 Mâlov, Denmark
Ethanol, absolute, min.99.85%	RT	100-9985.20L	Pure Science, PO Box 57-161 Mana, Porirua 5247
Ethylenediaminetetra-acetic acid disodium salt (EDTA, disodium salt), MW=372.2	RT	10093	BDH, now under the Merck Label. Merck New Zealand, 22 Hobill Avenue, Auckland 2104, New Zealand
Evans blue dye	RT	46160	Sigma-Aldrich New Zealand Ltd., P.O. Box 106-406 Auckland, 1030 New Zealand
Hepes Solution (1M)	4 – 5 °C	GIBCO15630-080	Life Technologies New Zealand, 18/24 Botha Road Penrose, Auckland 1135, New Zealand
Human chorionic gonadotrophin, Chorulon, 1500 IU freeze dried powder	Under 20°C, protect from light	Chorulon – 1500 IU	Intervet NZ, 12 Shakespeare Avenue, Upper Hutt 5018, New Zealand
Hyaluronidase, Type VI-S, lyophilized powder	-20°C	H3631	Sigma-Aldrich New Zealand Ltd., P.O. Box 106-406 Auckland, 1030 New Zealand
L-Glutamine, ($C_5H_{10}N_2O_3$), MW=146.1	RT	G-3126	Sigma-Aldrich New Zealand Ltd., P.O. Box 106-406 Auckland, 1030 New Zealand

Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$), MW= 246.48	RT	Ma0084	Scharlau, Gato Pérez, 3. Pol. Ind. Mas d'en Cisa E08181 Sentmenat, Barcelona, Spain
MEM Sodium Pyruvate Solution 100mM (Pyruvic Acid, sodium salt)	4 – 5 °C	GIBCO11360-070	Life Technologies New Zealand, 18/24 Botha Road, Penrose, Auckland 1135, New Zealand
MEM Essential Amino Acids Solution (x50)	4 – 5 °C	M5550	Sigma-Aldrich New Zealand Ltd., P.O. Box 106-406 Auckland, 1030 New Zealand
MEM Non-essential AA solution (x100)	4 – 5 °C	M7145	Sigma-Aldrich New Zealand Ltd., P.O. Box 106-406 Auckland, 1030 New Zealand
Penicillin-Streptomycin, liquid. 10,000 units penicillin and 10,000ug of streptomycin/mL, in 0.85% saline	- 20 °C	Invitrogen,15140-122	Life Technologies New Zealand, 18/24 Botha Road Penrose, Auckland 1135, New Zealand
Phenol red solution	RT	P0290	Sigma-Aldrich New Zealand Ltd., P.O. Box 106-406 Auckland, 1030 New Zealand
Pregnant mare serum gonadotrophin, Folligon, 5000 IU freeze dried powder	2 – 15°C, protect from light	Folligon – 5000 IU	Intervet NZ, 12 Shakespeare Avenue, Upper Hutt 5018, New Zealand
Potassium chloride (KCl), MW=74.55	RT	1049360500	Merck New Zealand, 22 Hobill Avenue, Auckland 2104, New Zealand
Potassium dihydrogen phosphate (KH_2PO_4), MW=136.09	RT	Po0260	Scharlau, Gato Pérez, 3. Pol. Ind. Mas d'en Cisa E08181 Sentmenat, Barcelona, Spain

Rodent diet, 18% protein, does not contain alfalfa, Teklad Global	RT	2018	Harlan Laboratories, Inc. 8520 Allison Pointe Blvd., Suite 400 Indianapolis, IN 46250, United States of America
Sterile mineral oil, mouse embryo tested	2 – 8°C, protect from light	M5310	Sigma-Aldrich New Zealand Ltd., P.O. Box 106-406 Auckland, 1030 New Zealand
Sodium bicarbonate (also known as Sodium hydrogen carbonate ($NaHCO_3$), MW= 84.01	RT	106329.05	Merck New Zealand, 22 Hobill Avenue, Auckland 2104, New Zealand
Sodium chloride (NaCl), MW=58.44	RT	102415K	BDH, now under the Merck Label. Merck New Zealand, 22 Hobill Avenue, Auckland 2104, New Zealand
Sodium hydroxide, reagent grade, > 98 %	RT	S5881	Merck Label. Merck New Zealand, 22 Hobill Avenue, Auckland 2104, New Zealand
Toluidine blue dye	RT	89640	Sigma-Aldrich New Zealand Ltd., P.O. Box 106-406 Auckland, 1030 New Zealand
2,2,2 – tribromoethanol, Br_3CCH_2OH	RT	T48402	Sigma-Aldrich New Zealand Ltd., P.O. Box 106-406 Auckland, 1030 New Zealand
Tertiary amyl alcohol, $CH_3CH_2C(CH_3)_2OH$, 98%	RT	152463	Sigma-Aldrich New Zealand Ltd., P.O. Box 106-406 Auckland, 1030 New Zealand
Tap water, Aa grade as per Ministry of Health, which reflects the processes used to treat drinking water and the network in which it is transported (New Zealand Ministry of Health, 2009).	-	-	Auckland City Council, Civic Building 1 Greys Avenue, Auckland 1010, New Zealand

Teklad Aspen Sanichips (bedding)	RT	7090A	Harlan Laboratories, Inc. 8520 Allison Pointe Blvd., Suite 400 Indianapolis, IN 46250, United States of America
Virkon	RT	-	Antec International Ltd, 47900 Fremont Blvd. Fremont, CA 94538

3.1.2.1. Preparation of in-house embryo culture and handling media

Preparation of embryo culture and handling media was a two-step process. Firstly, base media was prepared by dissolving the reagents described in Table 7 in 1000 mL of MilliQ water (18.2 MΩ.cm).

Table 7 Reagents used to prepare base media. Reagent name, molecular weight (MW), amount per 1000 mL of medium and concentration were summarised.

Reagent	Amount per 1000 mL medium	Unit	Concentration (M)
EDTA, disodium salt, MW=372.2	3.80×10^{-4}	g	1.02×10^{-5}
CaCl$_2$, anhydrous powder, MW=110.99	$2.50 \times 10\text{-}1$	g	$2.25 \times 10\text{-}3$
D-Glucose ($C_6H_{12}O_6$), MW=180.16	3.60×10^{-2}	g	2.00×10^{-4}
DL-Lactic Acid, sodium salt, MW=112.1	1.87	mL	1.00×10^{-2}
KCl, MW=74.55	1.86×10^{-1}	g	2.49×10^{-3}
KH$_2$PO$_4$, MW=136.09	4.76×10^{-2}	g	3.50×10^{-4}
L-Glutamine, ($C_5H_{10}N_2O_3$), MW=146.1	1.46×10^{-1}	g	1.00×10^{-4}
MgSO$_4$*7H$_2$0, MW=246.48	4.93×10^{-2}	g	2.00×10^{-4}
MEM Essential Amino Acids Solution (x50)	10.00	mL	-
MEM Non-essential AA solution (x100)	5.00	mL	-
MEM Sodium Pyruvate Solution 100mM	2.00	mL	2.00×10^{-4}
NaCl, MW=58.44	5.56	g	9.51×10^{-2}
Penicillin-Streptomycin liquid (1% solution)	1.00	mL	-
Phenol red solution (0.5% solution)	1.00	mL	-

To prepare embryo culture media, reagents described within Table 8, were dissolved in 100 mL of base media. Medium pH was then checked, which was expected to be between 7.8 – 7.9.

Table 8 Reagents used to prepare embryo culture media. Reagent name, molecular weight (MW), amount per 100 mL of medium and concentration were summarised.

Reagent	Amount per 100 mL medium	Unit	Concentration (M)
Bovine Albumin (BSA), Fatty Acid Free, lyophilized	0.10	g	(0.1%)
$NaHCO_3$, MW= 84.01	0.21	g	2.5×10^{-2}

To prepare embryo handling media, the reagents described in Table 9, were dissolved in 900 mL of base media. The pH of embryo handling media was then adjusted to 7.35 – 7.4 using a 5×10^{-4} M NaOH.

Table 9 Reagents used to prepare embryo handling media. Reagent name, molecular weight (MW), amount per 900 mL of medium and concentration were summarised.

Reagent	Amount per 900 mL medium	Unit	Concentration (M)
Bovine Albumin, Fatty Acid Free, lypholized	2.70	g	(0.3%)
Hepes Solution (1M) 238.3 g/L	4.89	g	-
$NaHCO_3$, MW= 84.01	3.15×10^{-1}	g	4.17×10^{-3}

Embryo culture and handling media were then sterile filtered using 0.22 µm Steritop-GP filter units attached 1 L Schott bottles and a vacuum pump. Media was divided into 500 mL aliquots in sterile culture flasks, which were sealed with parafilm and covered with aluminium foil. Both sterilisation and aliquoting of embryo culture and

handling media were performed within a Class II biohazard safety cabinet. Embryo culture and handling media was stored at 4 - 5 °C for up to two months.

Embry culture media was validated by a pH and embryo development test. A 5 mL aliquot of new media was placed in 5% CO_2 at 37°C overnight. The pH of the aliquot was checked the following day, and would need to fall within the acceptable range of 7.4 ± 0.5. Embryo quality and development following culture within the new batch of media, was also compared to the previous batch. To officially validate the new batch of culture media, at least 80% of embryos would need to reach a grade of 1A or higher by day-4.5 pc. Osmolarity was also tested for periodically, where 280 osmols was considered acceptable.

Embryo handling media was also validated an embryo development test. As with embryo culture media, both the previous and new batches of embryo handling media were compared. Embryos were incubated in 50 μL handling media microdrops, overlaid with sterile mineral oil, within a 37°C atmospheric incubator for a minimum of two hours. Embryos were then transferred to 50 μL culture media microdrops, also overlaid with sterile mineral oil, and cultured in 5% CO_2 at 37°C overnight. Embryo development was assessed the following day. To validate the new batch of embryo handling media, at least 80% of embryos would need to reach a grade of 1A or higher by day-4.5 pc.

3.1.2.2. Preparation of stock 10x Phosphate Buffered Solution

Stock 10x Phosphate Buffered Solution (PBS) was prepared by dissolving the reagents described in Table 10 in 1800 mL of MilliQ water (18.2 MΩ.cm). The pH was adjusted to 7.2 using 5×10^{-4} M NaOH. Solution was then brought up to 2000 mL.

Table 10 Reagents used to prepare stock 10x PBS. Reagent name, molecular weight (MW), amount per 2000 mL of MilliQ water (18.2 MΩ.cm) and concentration were summarised.

Reagent	Amount per 2000 mL	Unit	Concentration (M)
KCl, MW=74.55	4.00	g	2.68×10^{-2}
KH_2PO_4, MW=136.09	4.00	g	1.45×10^{-2}
NaCl, MW=58.44	160.00	g	1.37
$Na_2HPO_4 \cdot 2H_2O$, MW=177.99	23.00	g	6.46×10^{-2}

3.1.2.3. Preparation of stock solutions for insertion molecules: FSL-A, FSL-Le^Y, FSL-HA$_{high}$, FSL-HA$_{mid}$, and BioG

Molecules, which spontaneous inserted into cellular membranes, were either diluted in 1x PBS or embryo culture/handling media. Choice of diluent was dependent on desired working solution concentration. All working solutions were diluted in embryo culture media. When working solutions required a 1:10 dilution, 1x PBS was used to prepare stock solutions, which were stored indefinitely in 5 μL aliquots at 85 °C. Any working solutions that required less than a 1:10 dilution would result in a high 1x PBS to embryo media ratio, which is detrimental to embryonic health and development. For this reason, embryo culture or handling media was used as the diluent. Once reconstituted in diluent, stock solutions were stored at 4 – 5 °C up until the expiry date of the media used.

To prepare either type of stock solution, diluent was pre-warmed to 40°C. The desired amount of insertion molecule was weighed out and dissolved to 10 mg/mL. Stock solutions were then sterilised using 0.2 μm centrifugal sterile filter units at 10,000 *g* for three minutes. Stock solutions were stored as per above paragraph. Figure explains KODE™ technology in greater detail.

3.1.2.4. Preparation of stock and working solutions for non-insertion molecules: HMW-HA and BioG

A 5 mg/mL stock solution of HMW-HA was prepared in pre-warmed 1x PBS (40°C). This stock solution was stored at 4 – 5 °C for up to two weeks. A 1:10 dilution, with embryo transfer media as the diluent, resulted in a 0.5 mg/mL working solution. Prior to its use, the working solution was sterilised by 0.2 µm centrifugal filter unit, at 10,000 g for three minutes.

Stock solution concentration for BioG (made-in-house) was set to 20 mg/mL. Using 1x PBS as the diluent, 5 µL aliquots were stored at -85°C until required. A 1:4 dilution in embryo transfer or handling media prepared the working solution (5 mg/mL). Prior to its use, the working solution was sterilised by 0.2 µm centrifugal filter unit, at 10,000 g for three minutes.

3.1.2.5. Preparation of superovulatory drugs

Superovulatory drugs were prepared as per Blake (2003), and were considered standard practice for KODE Biotech Ltd. Dilute PMSG and hCG with 1x PBS to give a 50 IU/mL solution. Aspirate drugs into insulin syringes. Store at -20°C until required.

3.1.2.6. Preparation of progesterone antagonist

Mifepristone (RU486) was selected as the progesterone antagonist. A working solution of 2×10^{-5} g / 10 g was reconstituted in propylene glycol. The working solution was stored at 4 – 5 °C for the length of drug efficacy. Concentration selection was based upon Table 18.

3.1.2.7. Preparation of Avertin anaesthetic

Reagents, described in Table 11, were dissolved in 12.5 mL of MilliQ water (18.2 MΩ.cm) with the aid of a 58°C waterbath. Once fully dissolved, with no air bubbles present, anaesthetic was sterile filtered using a syringe driven 0.25 µm filter unit. Anaesthetic was stored at 4 - 5°C, in a 10mL sterile conical tube wrapped in aluminium foil, for up to two weeks.

Table 11 Reagents used to prepare Avertin anaesthetic. Reagent name, molecular weight (MW), amount per 10 mL of MilliQ water (18.2 MΩ.cm) and concentration were summarised.

Reagent	Amount per 10 mL	Unit	Concentration (M)
2,2,2 – tribromoethanol, MW= 282.76	1.22×10^{-1}	g	4.31×10^{-2}
Tertiary amyl alcohol, MW=88.15	0.25	mL	2.4%

3.1.2.8. Preparation of hyaluronidase

Immediately prior to embryo retrieval, hyaluronidase was diluted in embryo handling media to 300 IU/mL. The solution was then sterilised using syringe driven 0.25 µm filter unit and pre-warmed to 37°C. The hyaluronidase solution was made fresh each embryo retrieval and not stored.

3.1.2.9. Preparation of Evans blue dye

Evans blue dye was diluted in 1x PBS to give a 1% solution. A syringe driven 0.25 µm filter unit was used to sterilise the solution. Evans blue dye solutions were stored in sterile 10 mL conical tubes covered in aluminium foil, at 4 – 5 °C up to the expiry of the diluent.

3.1.3 Mouse colony

3.1.3.1 Ethics

Ethics approval was gained through the University of Auckland Animal Ethics Committee, and included the applications CR320 and CR622 for the duration of this research.

3.1.3.2 Animal management

All maintenance and breeding of mice was conducted within the Animal Unit of KODE Biotech Ltd, Auckland, New Zealand. Animal management and housing were in accordance with the recommendations of the University of Auckland Animal Ethics Committee.

Mice were housed in a semi-closed system, where temperature was regulated to 22 ± 2 °C, with atmospheric humidity. Fluorescent lighting was used for 12 hours a day (light phase), followed by 12 hours of darkness (dark phase). Air circulation was continuous, including ventilation and exhaust systems. Access to mice was limited to those immediately involved in experimentation (maximum of two people) and had the strict proviso of laboratory coats, overshoes, hairnets, and gloves. Masks were also used for any cleaning process.

Mice were contained within one of four possible cage systems (Table 12). Small cages (28 x 11 x 15 cm) were permitted to contain a maximum of six mice, which were less than 35 days old post-birth (pb), or a maximum of four adult female mice or three adult male mice, which were 35 days pb or older. Three large cage systems were available. The large cages, with flat top lids, (39 x 15 x 22 cm) catered for a maximum of nine recipient female mice, who were older than 35 days pb. In addition, this cage system was used for breeding tris, which included two adult female mice, and one adult male mouse, both older than 40 days pb (recognized as time of sexual maturity). Large cages with high-top lids (41 x 11 x 24 cm) presented the third cage system and were also used for breeding tris. The fourth cage system available was a large cage, with a modified high-top lid, used for restricted breeding with sterile (vasectomised) or fertile male mice (Section 3.2.3). Singularly houses male mice were spread throughout the colony to minimise the effect of the Lee-Boot effect (Van der Lee & Boot, 1955, 1956).

Teklad aspen sanichips (non-autoclaved) were used as bedding for all mouse cages. In addition, standard tap water (Aa grade as per New Zealand Minisitry of Health, 2009) and Teklad Global 18% rodent diet were supplied *ad libitum*. All cage and bottle systems were cleaned weekly or as needed with 1% Virkon solution. Bottle systems were rinsed with tap water a minimum of three times prior to reinstatement.

Table 12 Four possible cage systems utilised within mouse colony.

Cage description	Mouse description	Maximum number of mice permitted
Small	Weanling (day 18 pb)	6
	Prepubescent mice (days 18 - 35 pb)	6
	Adult mice (> day 35 pb)	4 females, 3 males
	Breeding Pairs (> day 35 pb)	2 (plus any litters)
	Fertile male mice	1
Large, flat top lid	Recipient female mice (> day 35 pb)	9
	Breeding tris (2x females and 1x male)	3 (plus any litters)
Large, high top lid	Breeding tris (2x females and 1x male)	3 (plus any litters)
Large, modified high top lid for RB	Vasectomised or fertile male mice	1

3.1.3.3 Breeding strategy

The breeding strategy for this research utilised two inbred foundational mouse strains, CBA/J and C57BL/6J; and a F1 hybrid strain, B6CBAF1/J, for experimentation (Figure 13). Foundational mouse strains were chosen for their efficiency to generate high numbers of experimental mice in a short space of time. The F1 hybrid strain was chosen for its well-understood background, ease of manipulation, and highly responsive nature to superovulatory drugs (prepubescent females only; Hogan, et al., 1994; Jackson Laboratories, 1988). Due to financial and space constraints associated with a small animal facility, no other mouse strains were investigated.

Foundational stock was purchased from The University of Auckland and was used to establish inbred CBA/J and C57BL/6J breeding pairs and tris. Genotypic drift was kept to a minimum as per the recommendations of Jackson Laboratories (2008). Every fourth litter, out of a maximum of six, was used to create new breeding pairs and tris for each mouse strain. In addition, foundational stock was completely replaced periodically times throughout the duration of this research. Replacement mice were obtained from the original source, The University of Auckland, to ensure genetic background remained consistent.
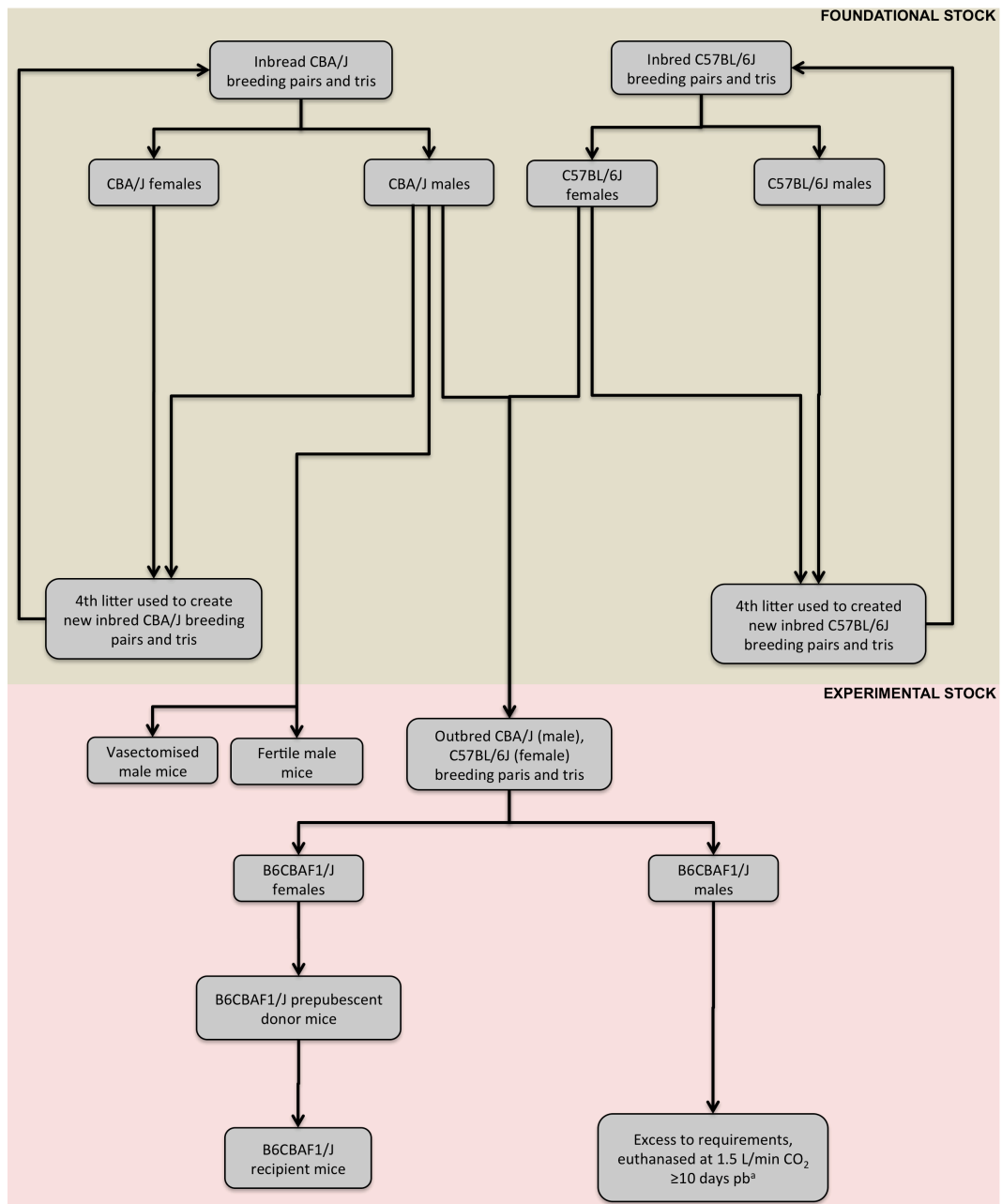
Figure 13 Breeding strategy and mouse utilisation. Foundational stock consisted of CBA/J and C57BL/6J mice. Experimental stock was produced by an outbred combination of CBA/J male mice and C57BL/6J female mice, resulting in B6CBAF1/J mice. Foundation stock was replaced three times throughout the duration of this research. Key: a, excess mice also included those surplus to requirements and/or had reached the end of experimentation, i.e. retired vasectomised male mice.

Resulting male pups of inbred CBA/J breeding pairs and tris were also used to supply experimental stock (Figure 13). Once sexually mature, CBA/J male mice were either assigned as vasectomised male mice for the production of pseudo-pregnant recipient mice; fertile male mice for production of donor embryos and natural pregnancy; or used to create outbred CBA/J x C57BL/6J breeding pairs or tris, which were used to

generate the remainder of experimental stock (B6CBAF1/J female mice). In contrast, female pups of C57BL/6J breeding pairs and tris were used to complete the outbred CBA/J x C57BL/6J breeding pairs or tris once sexually mature. If not required to create new inbred breeding pairs and tris, female CBA/J and male C57BL/6J mice were euthanased at 10 days pb using CO_2 at 1.5 L/min.

Similar to the foundational stock, outbred CBA/J x C57BL/6J breeding pairs or tris were not allowed to produce more than six litters. Female B6CBAF1/J pups were separated from their male siblings at the time of weaning, 18 days pb, and were made available for superovulatory stimulation from 21 – 30 days pb (embryo donors). Those female B6CBAF1/J mice not used to produce donor embryos were set aside for the recipient mouse pool, and entered active service from day 60. Female B6CBAF1/J mice, that did not come into estrus nor exhibit a seminal plug pc by day 100 pb, were euthanased at using CO_2 at 1.5 L/min. All remaining surplus mice, which included retired breeding pairs or tris (maximum of six litters reached), retired fertile or vasectomised male mice (plug rates less than 40%), and male B6CBAF1/J pups, were also euthanased with CO_2 at 1.5 L/min (≥10 days pb).

3.2 Methodology

3.2.1   Sterilisation of glassware and surgical equipment

All glassware and surgical equipment were soaked for a minimum of four hours in a 2% Extran® solution (diluent tap water). If necessary, items were gently scrubbed with a soft toothbrush to remove debris. Once soaking period was complete, all items were rinsed six times with tap water and six times with de-ionised water (conductivity ≤ 1 µS/m). The opening of all glass beakers or Schott bottles was covered with aluminium foil. All surgical equipment was placed in stainless steel containers, with container-lids firmly closed. Prepared glassware and surgical equipment was then placed in a 140ºC oven and heat-sterilised for 2-3 hours.

3.2.2   Production of vasectomised male mice

Vasectomies were performed on male CBA/J mice, which were 45 – 75 days old pb. Mice were anaesthised with avertin anaesthetic at 0.2 – 0.26 mL / 10 g body weight. Once unconscious, the lower abdomen was shaved with moustache clippers and swabbed using 70% ethanol (EthOH). A transverse incision was made through the

skin and peritoneum using sterile sharp fine surgical scissors. One at a time, each testicle was extracted using sterile fine rats-tooth forceps by grasping the fat-pad surrounding the testicle. The vas deferens was located by identifying the prominent blood vessel running parallel to it. An 8 – 10 mm section of the vas deferens was removed and cauterised using red-hot jewellers forceps (pre-heated using a Bunsen burner). Once the procedure was completed on both testicles, the peritoneum and skin incisions were closed using two suture stitches each.

Post-surgery, male mice were observed every two hours for the remained of the surgical day. Observations continued on a daily basis for a further seven days. Each post-surgical observation monitored mice for any significant sign of distress. Symptoms of distress included: a ruffled coat, hunched back, lack of movement, untoward restlessness, laboured respiration, lack of eating or drinking, diarrhoea or auditory distress. In addition, mice were monitored for post-surgical bleeding within two hours of surgery, and wound infection within 48 hours of surgery. Any significant concerns were discussed with consulting veterinarian. If necessary, vasectomised male mice were euthanased with CO_2 at 1.5 L/min.

Ten days post-surgery, the sterility of vasectomised male mice was assessed. Adult female mice, which were in estrus, were selected and placed with vasectomised male mice. If no litters resulted within 30 days of co-habitation, female mice were euthanased with CO_2 at 1.5 L/min, and their uterine horns examined for the presence of implants. If no implants were observed, vasectomised male mice were considered sterile and suitable to produce pseudo-pregnant recipient mice. When the seminal plug rate dropped below 40%, vasectomised male mice were retired and euthanased with CO_2 at 1.5 L/min.

3.2.3 Production of natural or pseudo-pregnant mice

Female B6CBAF1/J mice, which were 60 – 100 days old pb, were used to produce naturally pregnant and pseudo-pregnant recipient mice. At any one time, the pool of B6CBAF1/J female mice consisted of 50 – 60 mice, sourced from at least nine different litters. The external appearance of the vagina, used to identify estrus, formed the basis for selection of female mice. Table 13 describes the external vaginal appearance used to identify each stage of the estrus cycle as per Champlin et al. (1973).

To ensure visual inspection for estrus was accurate within this research, vaginal smears were performed daily for 15 days on 33 female B6CBAF1/J mice, which were also 60 – 100 days old pb. Mice were housed within four different cages and spread evenly throughout the colony. Vaginal smears were performed as per Hubscher et al. (2005). Vaginal smears identified that at any one time 17 – 20% of female mice were in estrus (5 – 6 days per cycle). In addition, 90% of female mice presenting the external vaginal appearance associated with estrus also had positive vaginal smears for estrus (data not presented). For this reason, vaginal appearance was used to identify estrus in female mice for the remainder of research.

Table 13 External vaginal appearance associated with the mouse estrus cycle as identified by Champlin et al, 1973.

Stage of Estrus Cycle	External vaginal appearance
Diestrus	Vagina has a small opening; tissues are blue and very moist.
Proestrus	Vagina is gaping; tissues are reddish-pink and moist; numerous longitudinal folds or striations are visible on both the dorsal and ventral lips.
Estrus	Vaginal signs are similar to proestrus, but the tissues are lighter pink and less moist, and the striations are more pronounced.
Metestrus 1	Vaginal tissues are pale and dry; dorsal lip is not as edematous as in estrus
Metestrus 2	Similar to Metestrus 1, but the lip is less edematous and has receded; whitish cellular debris may line the inner walls or partially fill the vagina.

To produce naturally pregnant mice, one to two B6CBAF1/J female mice, selected as per described above, were placed with CBA/J male mice of proven fertility. Coitus was thought to occur approximately at the mid-point of the dark phase, and was dependent on successful ovulation, which would normally occur one to three hours prior to the mid-point (Hogan, et al., 1994). The fertility status of CBA/J male mice was determined by closely monitoring the numbers of donor embryos produced when prepubescent B6CBAF1/J female mice were mated with the male mice. When 10 donor embryos or less resulted for more than three consecutive pairings (different prepubescent B6CBAF1/J female mice each time), CBA/J male mice were retired and euthanased with CO_2 at 1.5 L/min.

To produce pseudo-pregnant recipient mice, again one to two B6CBAF1/J female mice, selected as per described above, were placed with vasectomised CBA/J male mice of proven sterility. The sterility status of vasectomised male mice was determined as per Section 3.2.2. Two approaches were used to produce pseudo-pregnant recipient mice. These included: 1) unrestricted-coitus; and 2) restricted-coitus. Unrestricted-coitus was where CBA/J male mice had free access to B6CBAF1/J female mice for the entire dark phase. Restricted-coitus was where the length of time CBA/J male mice had access to B6CBAF1/J female mice was restricted. Restricted-coitus was achieved by placing B6CBAF1/J female mice in a holding cage adjacent to the main cage, which held a single CBA/J male mouse (Figure 14). At the mid-point of the dark phase, the sliding door opened between the two cages, which allowed the CBA/J male mouse access to B6CBAF1/J female mice.
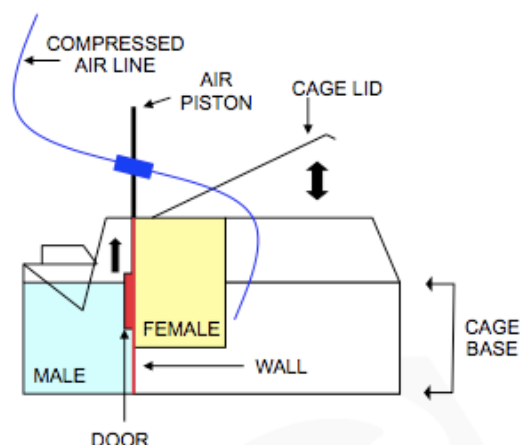
Figure 14 Cage design used for restricted-coitus. Modified high-top rat cage lid and base. Modified cage lid contains one holding cage and a Perspex wall, containing the male mouse a small section of the cage base. A small sliding door located on the Perspex wall separates the male and female mouse. At a specified time, a timer allows the release of compressed air, which travels down the line to the air piston, causing it to rise and open the sliding door, which allows male mouse access to female mouse.

The following morning, each B6CBAF1/J female mouse was examined for the presence of a seminal plug, which was identified by the filling of the vagina with seminal fluid and a rough exterior at the vaginal opening. The presence of a seminal plug indicated successful coitus, and B6CBAF1/J female mice were considered day-0.5 pc naturally pregnant or pseudo-pregnant (depending on the combination of mice). Female B6CBAF1/J mice that did not exhibit a seminal plug, or were excess to experimental requirements, were placed back into the recipient pool

(approximately 25%). For this reason, up to 25% of the recipient pool, at any one time, were potentially not virginal mice. Those B6CBAF1/J female mice that were older than 100 days pb were euthanased with CO_2 at 1.5 L/min.

As stated previously, at least nine different litters, providing a total of 50 – 60 B6CBAF1/J female mice, supplied the recipient pool. Although littermates represented up to 25% B6CBAF1/J female mice selected for estrus, littermates that became pseudo-pregnant were only used when numbers of available mice were limited and represented up to 15% of ET within this research.

3.2.4 Production of superovulated recipient mice

As per Section 3.2.3, B6CBAF1/J female mice were visually selected for estrus and kept aside overnight prior to superovulation. The following day, B6CBAF1/J female mice were injected intraperitoneally with 10 IU of PMSG (200 µL approximately at 4 - 5 IU / 10 g body weight). Forty-eight hours later, B6CBAF1/J female mice were injected intraperitoneally with 10 IU with hCG (200 µL at approximately 4 – 5 IU / 10 g body weight) and were immediately placed with vasectomised CBA/J male mice of proven sterility (non-restricted breeding, Section 3.2.3). The following morning superovulated B6CBAF1/J female mice were checked for the presence of seminal plugs. If successfully detected, superovulated B6CBAF1/J female mice were considered day-0.5 pc pseudo-pregnant and were used as ET recipients on day-2.5 pc.

It is worth noting that the value of 10 IU in a delivery volume of 200 µL (4 - 5 IU / 10 g body weight) was established by the combined recommendations of Blake (1997), Ertzeid and Storeng (2001) and Jackson Laboratories (1988). Superovulated recipient mice utilised within this research had a mean weight of 21.9 g, resulting in a delivery concentration of 4.6 IU / 10 g body weight. Although, this value was slightly higher than the recommendations of Jackson Laboratories (2 – 4 IU / 10 g body weight), 176 µL of each superovulatory drug would be required to achieve 4 IU / 10 g body weight. Due to the 20 µL increments of the syringes utilized, accurately delivering 176 µL would be difficult. Purchasing syringes or dispensing equipment with smaller increments would have increased the overall experimental cost. Nonetheless, pregnancy rates within this research (38%) were similar to those seen in Blake (1997), who observed a 44% pregnancy rate. For this reason, it was believed the small difference in IU would not cause any significant differences.

3.2.5 Production of glass handling and transfer pipettes and pipette aides

To minimise experimental cost, pipettes for handling and transfer of embryos were made using glass capillary tubes. One at a time, glass capillary tubes were held at both ends and the mid-point rolled over a hot Bunsen burner flame until glass was just softened. The two ends of the capillary tubes were pulled apart quickly to approximately shoulder width. The speed and the extent glass capillary tubes were pulled determined the final diameter of the pipette. Those stretched capillary tubes destined to become handling pipettes, were pulled slightly slower and less than shoulder width apart compared to their transfer counterparts.

One stretched glass capillary tube could be used to create two pipettes. This was achieved by breaking the stretched glass capillary tube at the mid-point. Each pipette was then shortened to approximately 80 mm and the tip passed quickly through a hot Bunsen burner flame to remove any sharp edges. Glass pipettes were then grouped by the bore diameter and hot-air sterilized. Transfer pipettes were typically smaller than handling pipettes. Their inner diameter would be wide enough to contain hatched mouse blastocyst embryos (just over 100 µm), but narrow enough to minimise their effect when passed through the uterine wall to aspirate donor embryos (note: uterine wall first punctured with a 29 G needle).

Pipette aids consisted of small-diameter silicone tubing, a syringe driven, 0.25 µm sterile filter unit and were controlled by the mouth. A small section of tubing (approximate 30 mm) was fitted to the lure-lock end of the filter unit, forming the mouthpiece. The filter unit would ensure technician protection from the specimen, and vice versa. A second section of tubing (approximately 750 mm) was fitted to the reverse side of the filter unit. The opposite end of the tubing would be used to hold either glass handling or transfer pipettes. The diameter of the second piece of tubing would need to be small enough to hold glass pipettes firmly in place.

3.2.6 Preparation of microdrops for embryo culture

Depending on experimental requirements, microdrops were prepared using either embryo handling or culture medium. Embryo handling media was used when embryos were exposed to atmospheric conditions for five minutes or longer. Embryo culture media was used when exposure to atmospheric conditions was less than five minutes.

All microdrops were prepared using aseptic technique within sterile 4-well IVF culture plates and adhered to the standards outlined in Hogan et al. (1994). Unless stated otherwise, all microdrops were 50 µL in volume and were overlaid with 900 µL of sterile mineral oil. Microdrops prepared with embryo handling media were pre-warmed within a 37°C atmospheric incubator for a minimum of 30 minutes prior to use. Microdrops prepared with embryo culture media were equilibrated for a minimum of 120 minutes within in a 5% CO_2 incubator, which was also set at 37°C.

3.2.7 Production of donor mouse embryos and culture

Eight to ten pre-pubescent B6CBAF1/J female mice, 21 – 27 days old pb, were selected. These mice were normally comprised of two sets of littermates. As per the recommendations of Jackson Laboratories (1988), pre-pubescent B6CBAF1/J female mice were injected intraperitoneally (IP) with 5 IU of PMSG (100 µL at approximately 4 IU / 10 g body weight). Injections were performed three to five hours prior to the start of the dark phase. Forty-eight hours later, pre-pubescent B6CBAF1/J female mice were IP injected with 5 IU of hCG (100 µL at approximately 4 IU / 10 g body weight). Individual pre-pubescent B6CBAF1/J female mice were immediately placed with individual CBA/J male mice of proven fertility.

The following morning pre-pubescent B6CBAF1/J female mice were examined for seminal plugs (Section 3.2.3). If seminal plugs were successfully identified, pre-pubescent B6CBAF1/J female mice were considered pregnant (day-0.5 pc). Subsequent days were described as day-1.5, 2.5, 3.5 and 4.5 pc.

As per Hogan et al. (1994), donor embryos were retrieved from successfully mated pre-pubescent B6CBAF1/J female mice on days 0.5 and 1.5 pc. For donor 1-cell embryos retrieved on day-0.5 pc, collection was performed from 21 hours pc (mid-point of the dark phase considered time = 0 hours). Each pre-pubescent B6CBAF1/J female mouse was humanely euthanased by cervical dislocation and abdomen swabbed with 70% EthOH. A small incision was made through the skin of the mid – lower abdomen with sterile surgical scissors. Tearing the skin sideways then opened the incision wider. With sterile sharp fine surgical scissors, an incision was then made as wide as possible through the peritoneum, so that skin and hair did not contaminate the dissection site. The uterine horns were then exposed in preparation for donor embryo collection.

Each uterine horn was extracted with sterile fine rats-tooth forceps and gently cleared of any surrounding connective tissue with the tips of sterile sharp fine surgical scissors. Dissection of each uterine horn was performed by cutting immediately below the ovary and mid-way down the uterine horn.

Both uterine horns were placed in 100 µL of 300 IU/mL sterile hyaluronidase solution, pre-warmed to 37°C, on a sterile Petri dish. Using the tip of a sterile 29 G needle (attached to an insulin syringe), the oviduct was torn either side of the donor embryos under a binocular dissection microscope. If necessary, the donor embryos were gently pushed out of the uterine horns. To dissociate cumulus cells, donor embryos were incubated for a further two to three minutes in the hyaluronidase solution on a 37°C heat-plate. Subsequent to three minutes, donor embryos were immediately washed three times in embryo handling media, pre-warmed to 37°C. Once washed, donor embryos were transferred to pre-equilibrated embryo culture microdrops and cultured *in vitro* in 5% CO_2 at 37°C until required.

For two to four-cell embryos, collected on day-1.5 pc, uterine horns, including the corresponding oviduct, were dissected as described above. However, embryos were retrieved by flushing 200 – 300 µL of handling media, pre-warmed to 37°C, through each oviduct (Hogan et al., 1994). Expelled embryos were immediately placed into pre-equilibrated embryo culture microdrops and cultured *in vitro* in 5% CO_2 at 37°C until required.

3.2.8   Grading of donor embryos

Embryo grading was used to indicate embryo health and development prior to ET. The grading system, sourced from Carter (2007b), was originally adapted from Gardner and Schoolcraft (1999). The following paragraphs will breakdown the grading system by early embryos, blastocyst and implantation competent embryos, and cellular structure and integrity.

Early embryos, prior to the blastocyst stage, were assigned a grade depending on the number of cells within the embryo. As described in Table 14, the first four possible grades were 2, 4, 8, and 16-cells. Those embryos immediately prior to the blastocyst stage (32 – 64 cells) were difficult to count and were, therefore, assigned M as their grade (morula).

Blastocysts and implantation-competent embryos were assigned grades based on size of the fluid filled blastocoel and whether or not the hatching process had begun (Table 15). These grades included: 5, 4, 3, 2, 1, Hg and Hd. Collapsed blastocysts, which had no blastocoel, received a grade 5 (collapsed blastocysts were only distinguishable from grade M embryos by the lack of an intact ZP). In contrast, those blastocysts that were fully expanded, immediately prior to hatching from their ZP, were assigned a grade 1. Once the process of hatching had started, blastocysts were assigned Hg (hatching) as their grade. Those blastocysts that had completely shed their ZP were graded as Hd (hatched).

Table 14 Grades used to describe early pre-implantation embryos.

Grading	Visual Factors
2-cell	2 cells
4-cell	3 – 4 cells
8-cell	6 – 8 cells
16-cell	12 – 16 cells
M	Compacted cells closely held together and difficult to count.

Table 15 Grades used to describe blastocysts and attachment competent embryos.

Grading	Description
5	No blastocoel
4	Blastocoel less than half the volume of the embryo
3	Blastocoel greater than half the volume of the embryo
2	Blastocoel completely fills the embryo
1	Blastocoel volume is larger than that of an early blastocyst
Hg	Hatching blastocyst
Hd	Fully hatched from zona pellucida

The final aspect considered when grading embryos prior to ET was their cellular structure and integrity. The potential grades included A, B and C, and were dependent on the amount of degradation observed and regularity of blastomere shape (Table 16).

Table 16 Grades used to describe the physical condition of pre-implantation embryos.

Grading	Description
A	No degenerative material, uniform blastomere cell size and shape
B	Minimal degenerative material, minor cytoplasmic fragmentation, minimal distortion of blastomere cell size and shape
C	Dark degenerative material, blastomere cells irregular in size and distorted shape

When the stage of embryo development and cellular structure and integrity were combined a two-way system was created (Table 17). Fifteen categories were generated, which recorded the poorest quality embryo as a 5C and the best quality embryo as a 1A.

Table 17 Combined grading system for both the stage of embryo development and the physical condition of pre-implantation embryos.

	Increasing developmental stage →				
Improving	5C	4C	3C	2C	1C
embryo	5B	4B	3B	2B	1B
quality	5A	4A	3A	2A	1A

3.2.9 Embryo transfer into mice

Section 3.2.9.1 will first detail the general surgical procedure used for ET into mice. This will be followed with a flow diagram explanation for replicate design for four different areas of research in Sections 3.2.9.2 to 3.2.9.5. These included: 1) the baseline investigation of a selection of optimal and suboptimal *in vivo* implantation

murine models (Sections 4.1 to 4.4); 2) the incidence of transmigration following murine ET (Section 4.5.1); 3) the assessment of the Optimal and Suboptimal model's ability to detect consequences of manipulated embryos (Sections 4.5 and 4.6); and 4) exploring the beginning of endometrial receptivity in pseudo-pregnant mice (Section 5.2).

Real-time development of donor embryos and recipient mouse endometrium was described as E"x" and R"x" days pc. Letters E and R represent the donor embryo and recipient mouse endometrium. Letter x represents the number days pc. Positive or negative symbols were added to describe delayed or advanced development compared to real-time. For example: day-3.5-day *in vitro* cultured embryos were described as E3.5(-) embryos. Recipient mice, where endometrium has matured for 2.5-days pc, were described as R2.5 recipient mice.

3.2.9.1   General surgical procedure

The extent of embryo-endometrial developmental synchrony within each optimal and suboptimal *in vivo* implantation murine model explored was dependent on their experimental design. The models included: 1) ET into SO recipient mice (advanced endometrium); 2) progesterone antagonist administration following ET (delayed endometrium); and 3) synchronous and asynchronous ET (shift of donor embryo and recipient endometrium developmental age at the time of transfer). How each model was generated and embryo manipulation, if utilized, was explained in more depth within Chapter 4. Surgical procedure adapted from Hogan et al. (1994).

Prior to ET, female B6CBAF1/J recipient mice were anaesthetised with Avertin anaesthetic at 0.2 – 0.26 mL / 10 g body weight. Once unconscious, a 1 – 2 cm square patch was shaved just above the left and right hips (two squares). Both shaved areas were swabbed with 70% EtOH. Using computer generated randomised lists, the uterine horn, order of treatments, and donor embryo selection were specified. Recipient mice were then placed into 90 x 100 mm Petri dishes in a dorsal/lateral position, specified by the computer generated lists, on a 37°C heat-plate.

Using sterile sharp fine surgical scissors, a small transverse incision was made through the skin and peritoneum, mid-way between the lowest rib and the top of the hip. The fat pad surrounding the ovary was located and gently drawn out of the body cavity using sterile fine rats-tooth forceps. A serrafine clamp was clasped to the fat pad and secured to blutac, located behind the middle of the spine. This meant that the top

quarter of the uterine horn, oviduct and ovary were now secure outside of the body cavity.

In preparation for ET, glass transfer pipette tips were pre-loaded with a series of sterile mineral oil, pre-warmed transfer media, and air bubble sections, to counteract the strong capillary action. Within the last section of media, five donor embryos, selected through the computer generated lists, were loaded in as little transfer media as possible.

A 29 G needle (insulin syringe) was used to puncture the uterine horn just below the utero-tubal junction. Pre-loaded glass transfer pipettes were inserted through the puncture to a depth of 4 – 5 mm, and donor embryos were gently aspirated into the uterine horn. The serrafine clamp was released, allowing the reproductive organs to retract back into the body cavity.

The peritoneum and skin incisions were then closed using two suture stitches each. To clean the surgical site of any blood, sterile 1x PBS was used to swab the area. This process was repeated with the opposite horn if required. As per Section 2.x, the production of vasectomised male mice, post-surgical observations were completed every two hours on the day of surgery and daily for a further seven days.

3.2.9.2 Replicate explanation for the baseline investigation of optimal and suboptimal *in vivo* implantation murine models

As illustrated in Figure 15, each ET into female B6CBAF1/J recipient mice represented one replicate. Within one experimental series (one week), a pool of pre-pubescent B6CBAF1/J female mice, which consisted of at least two litters, were superovulated and mated with CBA/J male mice of proven fertility (Section 3.2.7). On day-1.5 pc, donor embryos were retrieved and *in vitro* cultured in 5% CO_2 at 37°C until needed. A maximum of 25 embryos were placed into each 50 µL microdrop (Section 3.2.7). On the day of transfer, donor embryos were divided into replicates. Just prior to each transfer, 10 donor embryos of the highest quality were placed into microdrops containing the transfer medium (Section 3.2.8). Computer generated randomised lists determined the final uterine horn, order, embryo selection for each ET. Assessment of ET outcomes were performed day-17 pc as per Section 3.2.11.

3.2.9.3 Replicate explanation for the incidence of transmigration following murine ET

As illustrated in Figure 16, each ET into female B6CBAF1/J recipient mice represented one replicate. Within one experimental series (one week), a pool of pre-pubescent B6CBAF1/J female mice, which consisted of at least two litters, were superovulated and mated with CBA/J male mice of proven fertility (Section 3.2.7). On day-1.5 pc, donor embryos were retrieved and *in vitro* cultured in 5% CO_2 at 37°C until needed. A maximum of 25 embryos were placed into each 50 µL microdrop (Section 3.2.7). On the day of transfer, E3.5(-) embryos were divided into replicates. Just prior to each transfer, 10 E3.5(-) embryos of the highest quality were placed into microdrops containing the transfer medium (Section 3.2.8). Five E3.5(-) embryos were selected by random numbers and transferred into the left uterine horns of R2.5 recipient mice. As a control, media alone (< 0.5 µL) was transferred into the right uterine horns of the same recipient mice. At the time of experimentation, computer generated randomised lists, which assigned uterine horn, order and embryo selection, had not yet been introduced. Assessment of ET outcomes were performed days 8 - 10 pc as per Section 3.2.11.

3.2.9.4 Replicate explanation for investigating the ability of the Optimal and Suboptimal model to detect consequences of manipulated embryos

As in Section 3.2.9.2, each ET into female B6CBAF1/J recipient mice represented one replicate. As illustrated in Figure 17, production and *in vitro* culture of donor embryos was comparable to Section 3.2.9.2. Again on the day of transfer, donor embryos were divided into replicates. As described further in Section 3.2.9.3, the partial septum found within the mouse uterus appeared to limit the transmigration of embryos from one uterine horn to the other. For this reason, two groups of differently manipulated embryos were transferred into the same recipient mouse, which allowed that mouse to act as it's own control.

Each replicate, therefore, contained both manipulated (M) and unmanipulated (UM) treatment microdrops (50 µL). Just prior to each transfer, 10 donor embryos of the highest quality were placed into each of these microdrops and were incubated as specified in Sections 4.5 and 4.6. The molecules, diluent, and media used for transfer were given in greater depth in each results section (Sections 4.5 and 4.6). Computer generated randomised lists determined the final uterine horn, order, embryo selection

for each ET. Embryos were transferred into recipient mice no more than 30 minutes post-manipulation. Assessment of ET outcomes were performed day-17 pc as per Section 3.2.11.

3.2.9.5 Replicate explanation for exploring the beginning of endometrial receptivity within pseudo-pregnant recipient mice

The experimental design utilized to explore the beginning of endometrial receptivity was intricate and focused on strict time frames. Donor embryo production and *in vitro* culture leading to ET was comparable to Section 3.2.9.2. However, replicates were grouped by the time-points assessed for the beginning of endometrial receptivity (88, 90 or 92 hours pc). Due to the strictly controlled times of ET and assessment, only one recipient mouse could be assigned to each assessed time-points per experimental series (Figure 76 and Figure 77). For this reason, experimental series one represented the first replicates for 88, 90 and 92 hours pc (Figure 18). Experimental series two represented the second replicates for 88, 90 and 92 hours pc, and so on. All other features of a replicate were comparable to that of Section 3.2.9.2.
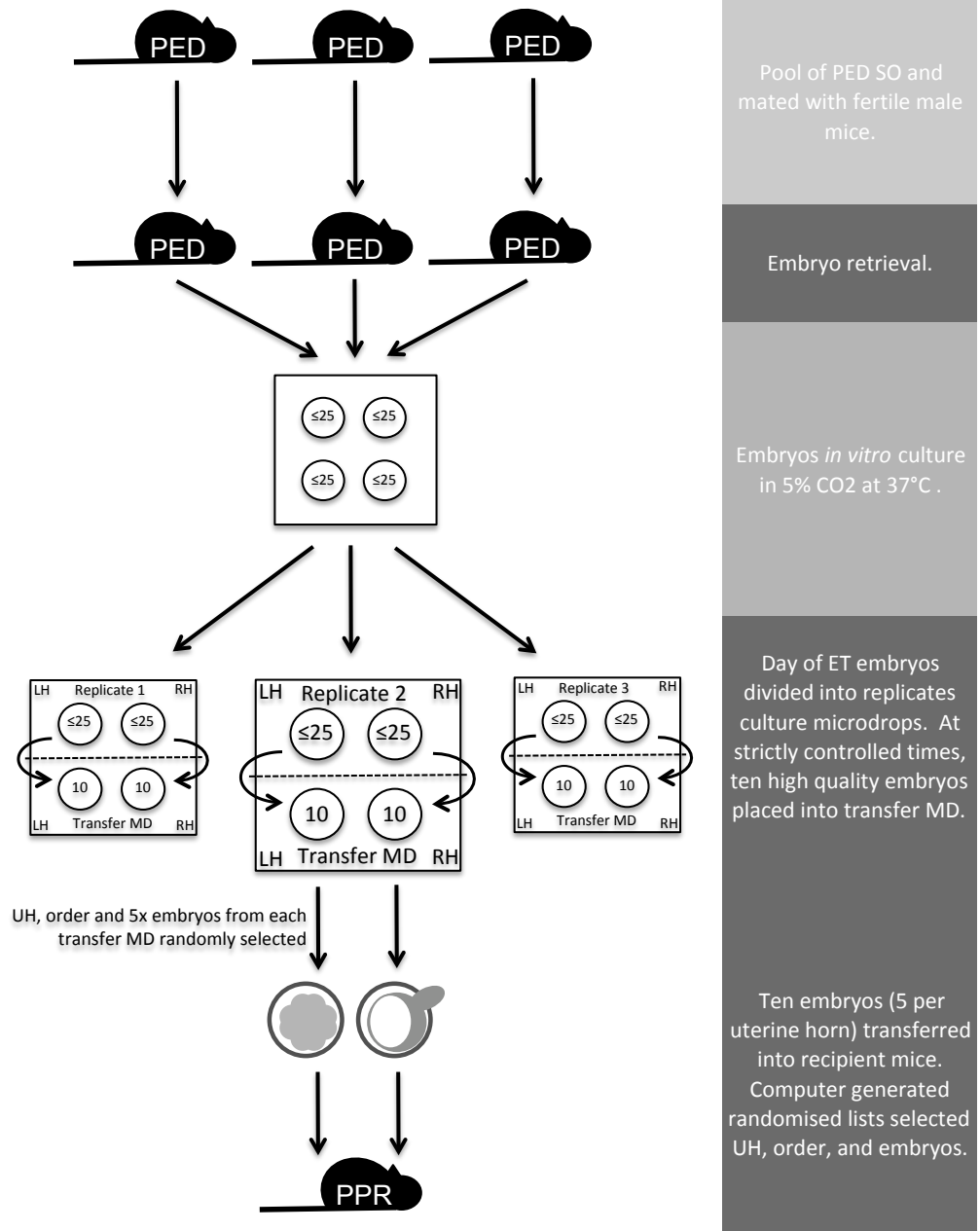
Figure 15 Example of replicates within one experimental series for the baseline investigation of optimal and suboptimal *in vivo* implantation murine models. Numbers inside the circles are the numbers of embryos per 50 μL embryo culture media microdrop. Just prior to ET, 10 high quality embryos per uterine horn were placed into embryo transfer media microdrops. Transfer media was embryo culture media, unless otherwise stated. High quality embryos, depending on the day of transfer, included M, 2A, 1A, HgB and HdB embryos. Key: PED, pre-pubescent embryo donor; SO, superovulated; ET, embryo transfer; LH, left uterine horn; RH, right uterine horn; MD, microdrops; UH, uterine horn; PPR, pseudo-pregnant recipient.
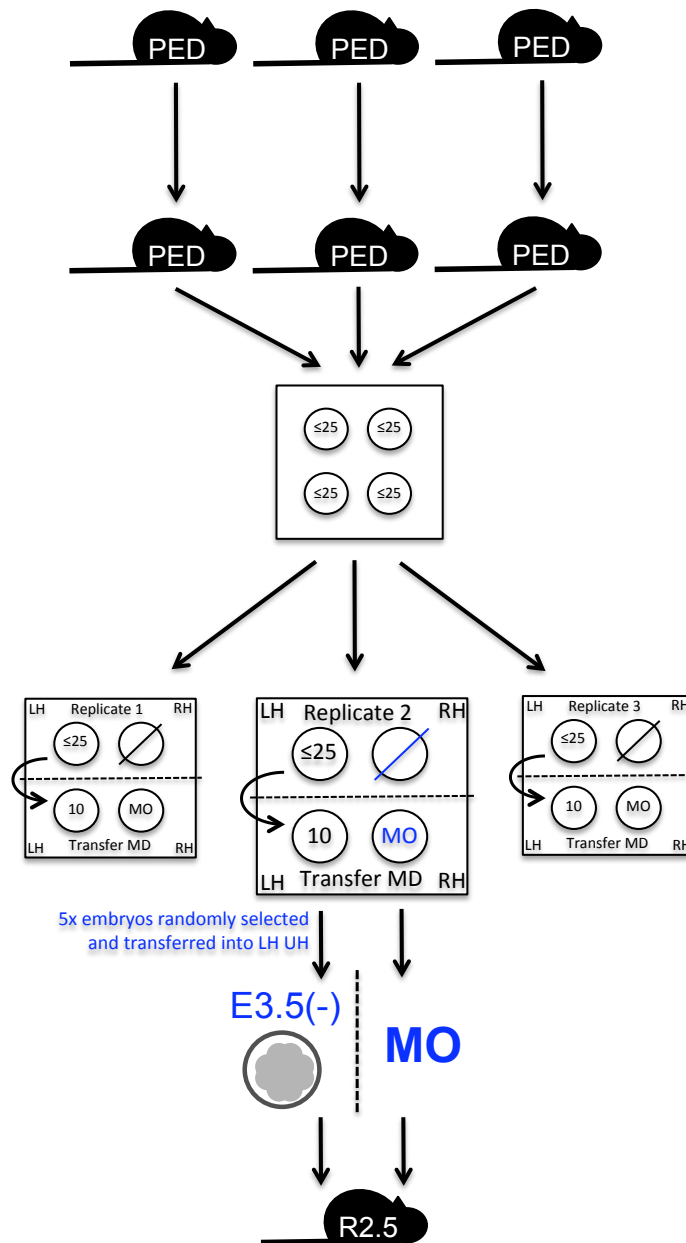
Figure 16 Example of replicates within one experimental series for the incidence of transmigration following murine ET. High quality embryos included: M, 2A and 1A. Differences are highlighted in blue, otherwise all other features as per Figure 15. Key: MO, media only; E3.5(-), *in vitro* cultured day-3.5 pc donor embryo; R2.5, day-2.5 pc pseudo-pregnant recipient.
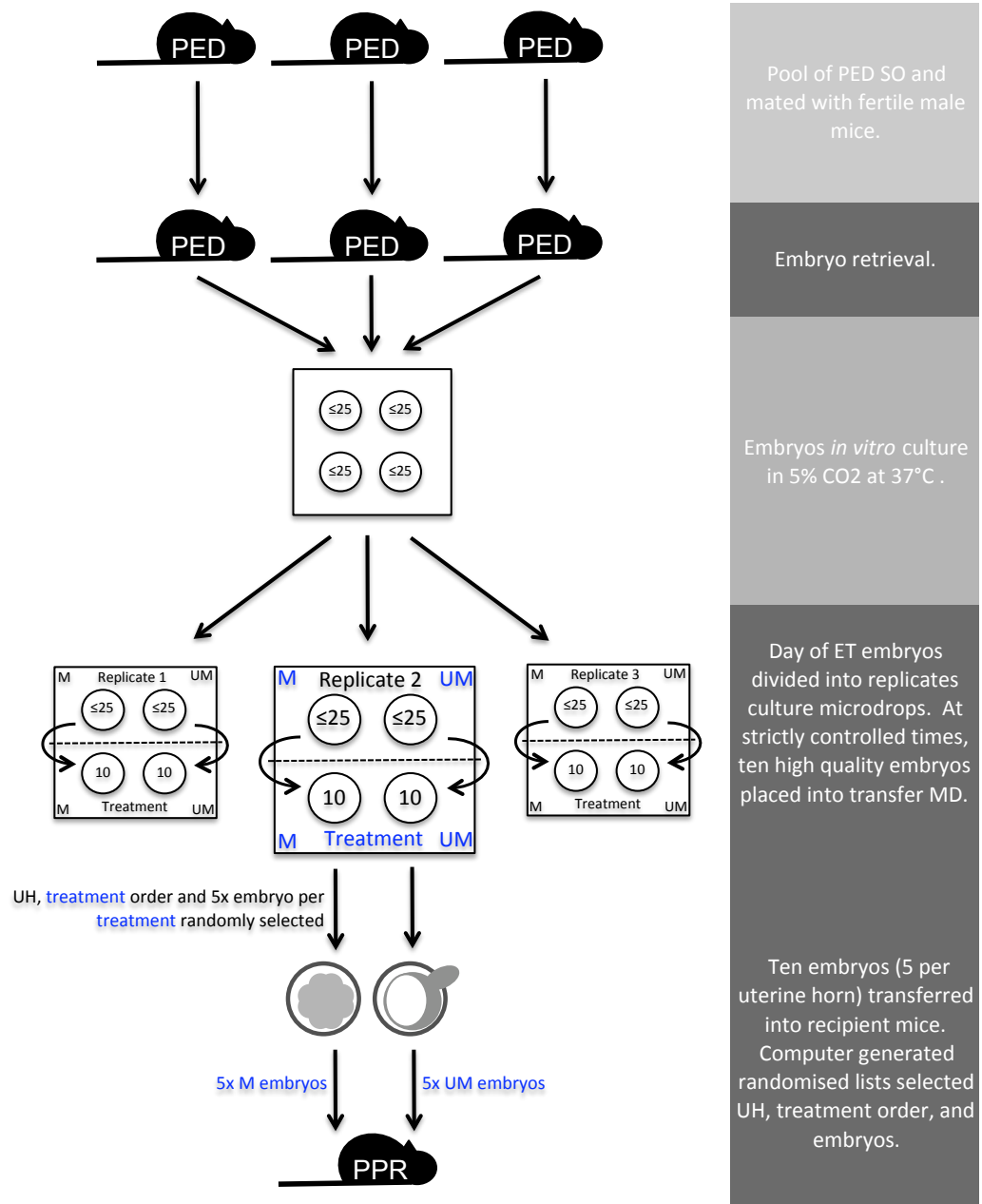
Figure 17 Example of replicates within one experimental series for investigating Optimal and Suboptimal Model's ability to detect consequences of manipulated embryos. High quality embryos included: M, 2A and 1A embryos (Optimal model); and HgB and HdB embryos (Suboptimal model). Differences are highlighted in blue, otherwise all other features as per Figure 15. Key: M, manipulated; UM, unmanipulated.
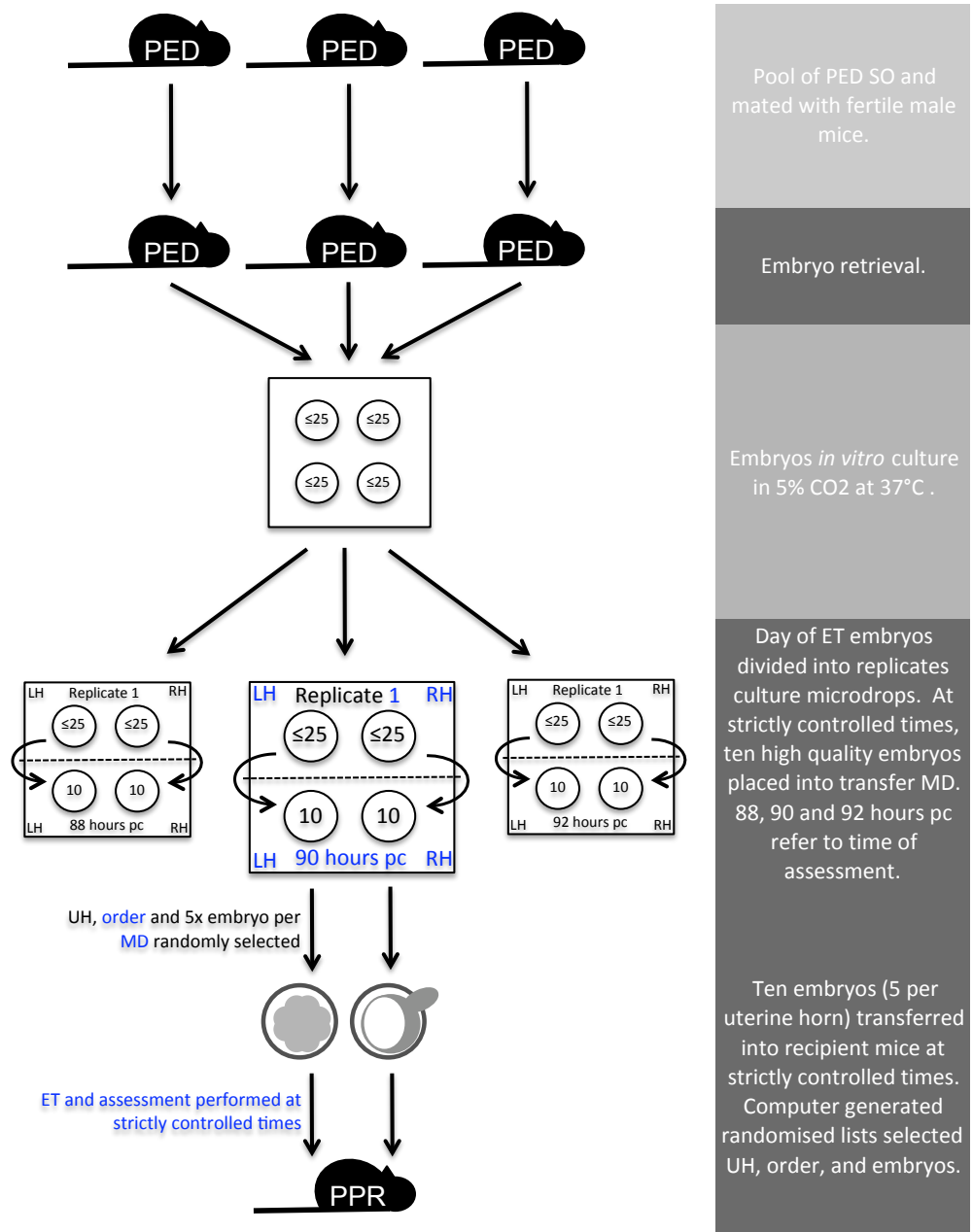
Figure 18 Example of replicates within one experimental series for investigating the beginning of endometrial receptivity. Due to strictly controlled times of ET and assessment, only one replicate (recipient mouse) could be assigned to each assessed time-points per experimental series (Figure 76 and Figure 77). Differences from Figure 17 are highlighted in blue, otherwise all other features as per Figure 15.

3.2.10 Progesterone antagonist administration following ET

Production of female B6CBAF1/J recipient mice, donor embryos and ET were completed as per Sections 3.2.3, 3.2.7 and 3.2.9 for the Optimal model (Section 4.4). At approximately 87 hours pc, one day post-ET, female B6CBAF1/J recipient mice were subcutaneously injected with RU486, a progesterone antagonist. The literature suggested that endometrial receptivity, thus implantation, began at approximately 92 hours pc (Finn & McLaren, 1967; McMaster, et al., 1993). Therefore, administration of RU486 was implemented just prior to implantation. Using propylene glycol as the diluent, concentration was set at 2×10^{-5} g / 10 g of body based upon the literature summarised in Table 18. Subcutaneous injection was delivered into the scruff of the neck. Note the half-life of RU486 is recognized as 12 – 24 hours within rodents (Tébar, et al., 1996).

Table 18 Experimental variables and resulting data associated with individual studies that utilised progesterone antagonists to generate suboptimal *in vivo* implantation murine models. Note: all data was collated from naturally pregnant mice.

Researchers	Goa et al. 2001	Liu et al. 2003	Liu et al. 2008
Mouse strain	Kunming	Kunming	Kunming
Mouse number	6 - 7/group	20/group	20/group
Time of assessment (days pc)	5.5	7.5	3.8
Time of injection (days pc)	0.5 and 1.5[a]	3.5	3.3

RU486 administered (g/10g body weight)	Mean number of implants per mouse		
0.0	14.3	14.22	9.94
3.0E-06			1.4
1.5E-05		10.7	
1.8E-05		10.5	
2.5E-05		8.5	
3.0E-05		6	
4.0E-05	7.3	0	
1.2E-04	0.5		

a = Injections both delivered in the morning of specified days.

3.2.11 Assessment of embryo transfer outcomes

All procedures are adapted from Hogan et al. (1994) and Psychoyos (1961).

3.2.11.1 Assessment on days 3 – 7 pc

For assessment of ET outcomes on days 3 – 7 pc, B6CBAF1/J female recipient mice were anaesthetised with Avertin anaesthetic at 0.2 – 0.26 mL / 10 g body weight. Once unconscious, mice were placed in dorsal recumbency and the area from the base of the neck towards the right hand front limb was shaved. Seventy percent EthOH was then used to swab the shaved area. Lateral to the anterior end of the sternum, a small incision was made through the skin using sterile sharp fine surgical scissors. Blunt dissection was used to tear the incision up to 1 cm in length. Using sterile sharp fine surgical scissors, subcutaneous fat was cleared to expose the pectoral muscle and subclavian sinus underneath the muscle.

A 1 mL syringe and 25 G needle were pre-loaded with 200 µL of sterile 1% Evans blue dye solution. Pinching the pectoral muscle using a pair of sterile fine rats-tooth forceps, the preloaded syringe and needle were gently inserted through the muscle at a 45° angle until the needle tip could be visualized within the subclavian sinus. The dye solution was slowly aspirated over a period of one minute. Immediately after needle withdrawal, the pectoral muscle was pinched to insure muscle closure and prevent bleeding from the subclavian sinus.

Following a 15 minute incubation to allow dye circulation, B6CBAF1/J female recipient mice were humanely euthanased by cervical dislocation. With sterile sharp fine surgical scissors, a small incision was made through the skin of the mid-lower abdomen. Tearing the skin sideways opened the incision wider. A second incision was made through the peritoneum to access the reproductive organs. Using the back of the scissors, the intestine was pushed to one side and the uterine horns excised.

Under binocular dissecting microscope excised uterine horns were assessed for the number of blue-bands. The presence of blue bands indicated decasualization and the implantation site. The intensity and coverage of each band was graded as per Table 19.

Table 19 Grades used to describe the intensity of blue-bands indicating implantation.

Grading	Description
1+	Very faint, but present blue-band covering less than 50% of uterine horn circumference.
2+	Faint blue-band covering greater than 50%, but less than 75% of uterine circumference.
3+	Clear blue-band covering 75% or more of uterine circumference.

3.2.11.2 Assessment on days 8 – 10 pc

Female B6CBAF1/J recipient mice were humanely euthanased with CO_2 at 1.5 L/min. Uterine horns were excised as per Section 3.2.11.1. As described in Section 3.1, within natural mouse pregnancy or following ET, embryos will either implant or fail to implant. Those embryos that successfully implant will then give rise to a viable fetus or non-viable fetus (resorptions and intra-uterine death post-implantation). Embryos that fail to implant quickly degenerate within the refractive endometrium, resulting in a loss (pre-implantation loss). For this reason, the numbers of implants, viable and non-viable fetuses, and losses were recorded for both uterine horns.

3.2.11.3 Assessment on days 16 – 17 pc

Like in Section 3.2.11.1, female B6CBAF1/J recipient mice were humanely euthanased with CO_2 at 1.5 L/min. Uterine horns were exposed as per Section 3.2.11.1. Each fetus was immediately pinched with serrated forceps to check for viability. The numbers of implants, viable and non-viable fetuses, and losses were recorded. Dissection was then paused for a minimum of three minutes to allow for fetal unconsciousness. Each uterine horn was excised and placed on a power towel to remove excess blood. Excised uterine horns were then placed in a Petri dish or on a laminated gird layout, with the left and right uterine horns clearly labelled for photography. Each fetus and their corresponding placenta were excised from the uteri and fetal membrane removed. After excised fetuses and placentae were cleared of excess blood, each were weighed and placed in another Petri dish or on a laminated gird layout for photography. Fetuses were arranged in chronological order from closest to the cervix (bottom) leading to the closest to the oviduct (top).

4 Results – Optimal and suboptimal *in vivo* implantation murine models

Standardization of *in vivo* implantation murine models, which were suitable to explore the initial potential of embryo manipulation as an IVF-intervention, required both consistent implantation rates and an ability to detect consequences of that intervention. Part of this process was to gain an understanding of fundamental knowledge surrounding murine implantation rates. For this reason, the outcomes on day-17 pc of natural mouse pregnancy were compared with the ET outcomes of a selection of optimal and suboptimal *in vivo* implantation murine models, whose embryo-endometrial developmental synchrony had been altered in some way. The models included: 1) ET into SO recipient mice (advanced endometrium); 2) progesterone antagonist administration following ET (delayed endometrium); and 3) synchronous and asynchronous ET (shift of donor embryo and recipient endometrium developmental age at the time of transfer). Brief interpretations are provided at the end of each section, which were used to select the most suitable optimal and suboptimal *in vivo* implantation murine models. Selected models were then assessed for their ability to detect consequences of manipulation embryos (Section 3.5 and 3.6).

4.1 Natural mouse pregnancy

The primary focus of this research was to develop an understanding of the impact of altered embryo-endometrial developmental synchrony on optimal and suboptimal implantation rates. Due to the lack of clarity within the literature, it was important to not only understand the outcomes of natural mouse pregnancy (Section 4.1), but also those associated with a selection of optimal and suboptimal models (Sections 4.2 to 4.4). An understanding of natural mouse pregnancy within the context of this research is addressed within this section.

Following natural mouse fertilization, an embryo will either implant or fail to implant (Figure 19). A successfully implanted embryo can have one of two outcomes: become a viable fetus; or be lost post-implantation as a non-viable fetus, which include both resorptions and intra-uterine death.

Those embryos that fail to implant prior to the end of endometrial receptivity, result in a loss (pre-implantation loss). Subsequently, the embryo quickly degenerates within the refractive endometrium. Note that although the mechanism behind pre/post-implantation loss is not crucial within this research, it is possible embryonic chromosomal abnormalities were responsible for these losses (Voullaire, Wilton, McBain, Callaghan, & Williamson, 2002).
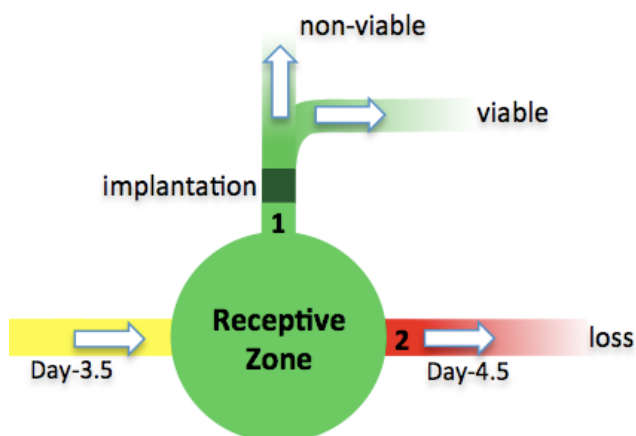
Figure 19 Schematic representation of the possible natural mouse pregnancy outcomes. On day-3.5 post-coitus (pc), the developing embryo reaches the receptive zone, representing the finite period of endometrial receptivity. Prior to the end of the receptive zone, the embryo will either successfully implant (1), or fail to implant (2). Successfully implanted embryos result in a viable or non-viable fetus. Only viable fetuses are capable of a live birth. Non-viable fetuses are lost post-implantation. Embryos that fail to implant, quickly degenerate within the refractive endometrium, leading to a loss.

To clearly understand natural mouse pregnancy within the context of this research, naturally pregnant female mice, of the same strain as the optimal and suboptimal models, were assessed for the presence of implants, viable and non-viable fetuses, and losses on day-17 pc. The B6CBAF1/J mouse strain is considered a good breeder by Jackson Laboratories, achieving 3 – 7 pups per litter (nd). Therefore, implantation rates were expected to meet these minimum values. Implantation rates within this section through to Section 4.4, and later in Chapter 5, were expressed as the mean numbers of implants per recipient mouse. In addition, the mean fetal and placental weights, and gross morphology and development were assessed. Data was used as a baseline for comparison to each assessed model within this research.

4.1.1 Methodology overview:

A total of 18 B6CBAF1/J female mice were used to assess the outcomes of natural pregnancy. This translated to nine replicates spread over three experimental series (three weeks). As per Section 3.2.3, naturally pregnant B6CBAF1/J female mice were produced through their unrestricted-coitus with CBA/J male mice of proven fertility. The presence of seminal plugs the following morning indicated successful coitus and represented day-0.5 pc. Naturally pregnant B6CBAF1/J female mice were then housed individually until the day of autopsy. No more than 15% of naturally pregnant B6CBAF1/J female mice were littermates. Animal management and breeding strategies applicable to Section 4.1 are detailed within Section 3.1.3.3.

On day-1.5 pc, nine naturally pregnant B6CBAF1/J female mice were euthanased and their uteri excised. Dissecting technique and embryo retrieval is detailed further within Section 3.2.11. Each horn was flushed using embryo handling media and the number of resulting two-cell embryos counted. The second group of nine naturally pregnant B6CBAF1/J female mice were euthanased seventeen days pc, and their uterus excised as per Section 3.2.11. Photos were taken of intact uteri, followed by the excised fetuses and their corresponding placentae. The numbers of viable and non-viable fetuses was recorded for each uterine horn. Each fetus and placenta was weighed, and gross morphology and development were assessed visually. The difference between the total numbers of embryos retrieved on day-1.5 pc and the total numbers of implants observed on day-17 pc were used to estimate the number of embryos that resulted in a loss. Standard deviation was used to demonstrate mouse-to-mouse variation.

4.1.2 Results:

Seventeen days pc, naturally pregnant mice resulted in a mean implantation rate of 9.0 implants per mouse (SD±0.9; Table 20,). All mice resulted in 8 or more implants per mouse (Figure 20). Of those implants, a mean of 8.7 (SD±1.2) were viable and 0.3 (SD±0.7) were non-viable. The mean fetal and placental weights on day-17 pc were 1.0 g (SD±0.07) and 0.1 g (SD±0.01), respectively. A mean fetal:placental weight ratio of 8.5 (SD±1.0) resulted. Normal gross morphology and development can be seen in Figure 21.

Table 20 Natural mouse pregnancy outcomes. Mean numbers of implants, viable and non-viable fetuses, and mean fetal and placental weights in nine naturally pregnant mice (day-17 pc). The numbers of embryos retrieved from a duplicated group of mice were used to estimate the mean number of losses (day-1.5 pc).

Outcome	Unit	Mean per mouse (±SD)	Total
Implants viable + non-viable fetuses	n	9.0 (±0.9)	81
Viable fetuses	n	8.7 (±1.2)	78
Non-viable fetuses	n	0.3 (±0.7)	3
Losses	n	0.0	0
Fetal weight	g	1.0 (±0.07)	-
Placental weight	g	0.12 (±0.01)	-
Fetal:placental weight ratio	-	8.5 (±1.0)	-
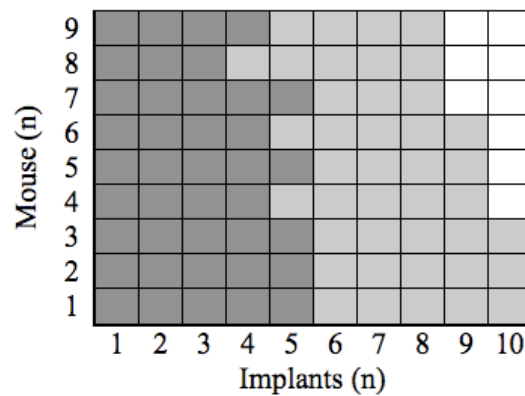
Figure 20 Numbers and location of implants within nine naturally pregnant mice. Each row represents one recipient mouse, arranged from highest to lowest numbers of implants. The numbers of shaded squares indicate the numbers of implants on day-17 pc. Implant location is indicated by either dark-grey squares (left uterine horn) or light-grey squares (right uterine horn). All mice resulted in 8 or more implants per mouse.
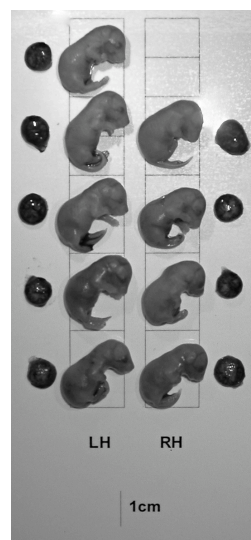
Figure 21 An example of gross morphology and development on day-17 pc of natural mouse pregnancy. Position of fetuses and placentae on grid indicated position within uterine horns. Fetuses were placed on the grid in chronological order from closest to the cervix (bottom of grid) leading to those closest to the oviduct (top of grid).

4.1.3   Interpretation:

Natural mouse pregnancy resulted in a mean implantation rate of 9.0 implants per mouse (Figure 22). Implants were detected in all assessed mice, ranging from 8 - 10 implants per mouse. Nine or more implants were observed in 67% of mice. Not only were the majority of implants viable (97%), but also no losses were observed. The mean fetal (1.0 g) and placental (0.12 g) weights, and gross morphology and development provided expected outcomes if normal implantation and development were to occur. It is worth noting that a natural variation in the moment of ovulation is expected between mice, and could account for the small variability observed in fetal and placental weights day-17 pc.
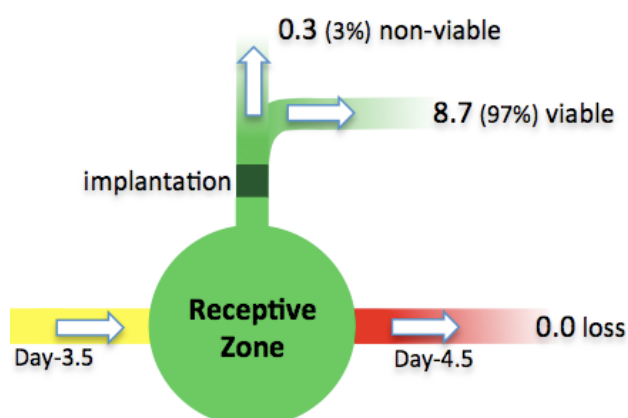
Figure 22 Schematic representation of mean natural mouse pregnancy outcomes. Natural mouse pregnancy resulted in a mean implantation rate of 9.0 implants per mouse. Of those successfully implanted embryos, a mean of 8.7 (97%) led to viable fetuses and 0.3 (3%) led to non-viable fetuses. No embryos resulted in a loss.

4.2 ET into superovulated recipient mice

Administration of superovulatory drugs within fresh IVF cycles is thought to accelerate human endometrial maturation (Bourgain & Devroey, 2003; Kelley, et al., 2006; Lee, et al., 2008; Oehninger, 2008). As a result, the endometrium is advanced for its age. A similar pattern has also been observed in superovulated adult female mice, which can result in suboptimal implantation rates (Figure 23). However, implantation rates varied from 7 - 62% between different research groups (Ertzeid & Storeng, 2001; Fossum, et al., 1989; Kelley, et al., 2006; Van der Auwera & D'Hooghe, 2001). Numerous parameters appeared to contribute to the lack of a standardised model. These could include: recipient mouse strain, age of recipient and embryo donor mice, day of embryo retrieval, numbers of embryos transferred or even colony diet. As a result, Section 3.2 assesses the ability of superovulated recipient mice to generate suboptimal implantation rates subsequent to ET.

Two criteria were used as a foundation to interpret data and thus determine the models suitability as a suboptimal model. Firstly, the model's impact on implantation needed to prevent implantation (pre-implantation loss) rather than disrupt or damage implantation (post-implantation loss). If implantation were prevented, increased numbers of losses would be observed. If implantation was disrupted or damaged, increased numbers of non-viable fetuses would be exhibited, potentially leading to an optimal implantation rate.

Secondly, the model would need to achieve an approximate mean of 6 implants per recipient mouse for every 10 embryos transferred (± 1 implant). This level of implantation was approximately 70% of natural mouse pregnancy within this research (9.0 implants per recipient mouse, SD± 0.9). Models exhibiting this level of implantation would demonstrate implantation capability. Within the current experimental design, it would be difficult to determine whether low numbers of implants were a product of recipient mice being incapable of implantation or whether the technique, used to generate the model, was itself responsible. If resources and funding were unlimited, which is often not the case for small research facilities; other techniques could be implemented to determine implantation capability of mice. For example microarrays could be used to examine gene expression profiles of recipient endometrium and compare them to profiles associated with normal implantation (Reese, et al., 2001).
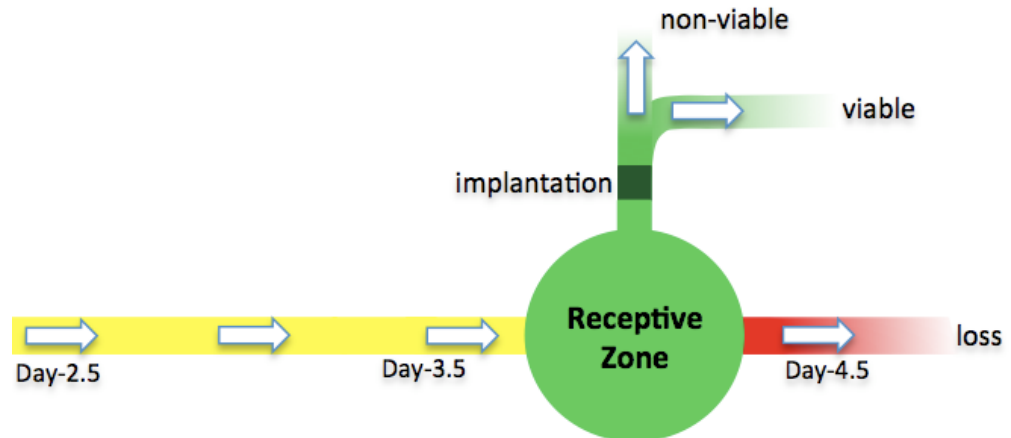
Schematic A – Normal endometrial maturation in non-superovulated recipient mice
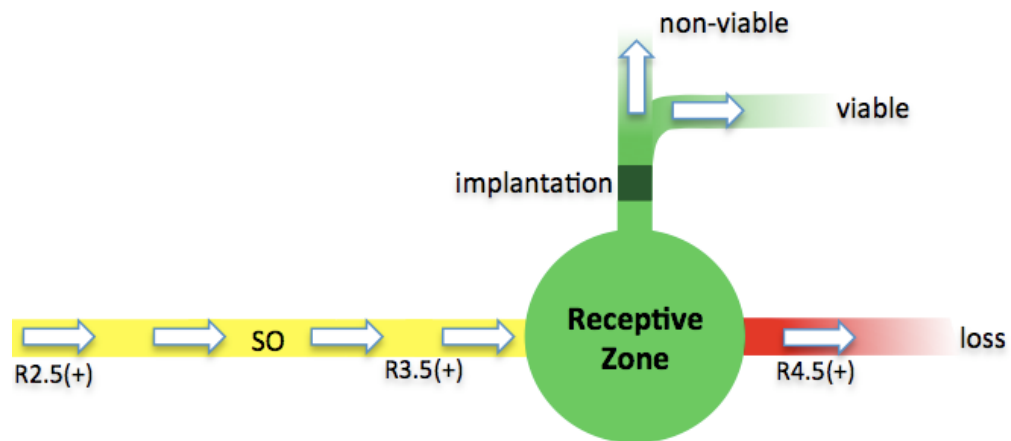
Schematic B – Advanced endometrial maturation in superovulated (SO) recipient mice

Figure 23 Schematic representation of the impact of superovulatory drugs on endometrial maturation. Schematic-A illustrates normal endometrial maturation leading to the receptive zone. Schematic-B illustrates the accelerated pathway to reach the receptive zone within superovulated recipient mice. As a consequence, the endometrium is more advanced than its real-time age, indicated by a positive symbol (+).

Models that exhibited an approximate mean implantation rate of 6 implants per recipient mouse (± 1 implant) could also clearly demonstrate any positive consequences of embryo manipulation, an IVF-intervention. Any significant increases in implantation, which could see natural levels of implantation restored (20 - 30% increase), could be achieved while keeping the numbers of experimental mice to a minimum. Thus in keeping with the three Rs: refinement, reduction and replacement. As stated in the previous paragraph, models that exhibited low levels of implantation

raised questions surrounding implantation capability. Thus, the numbers of experimental mice required would need to increase in order to demonstrate any significant increases in implantation. In contrast, models that exhibited high numbers of implants (>9.0 implants per mouse for every 10 embryos transferred), already achieved natural levels of implantation. To demonstrate a significant 10% increase would require substantially higher numbers of experimental mice compared to a 20 - 30% increase. Therefore, models that exhibited mean implantation rates outside of the 6 implants per recipient mouse (± 1 implant) would increase the overall experimental cost, time associated, and were less in line with the three Rs.

The criteria described above were also used to interpret the findings associated with administration of progesterone antagonists following ET, and synchronous and asynchronous ETs that achieved low levels of implantation (Sections 4.3 and 4.4).

4.2.1   Methodology overview:

Embryos were produced and collected from pre-pubescent donor B6CBAF1/J mice on day-1.5 pc (Section 3.2.7; Figure 24). Embryos were then *in vitro* cultured in 5% CO_2 at 37°C for two days. As per Section 3.2.3, B6CBAF1/J female mice, which were 60 - 100 days old pb, were visually selected for estrus from the recipient mice pool. The following day, the selected recipient mice received 10-International Units (IU) of follicle stimulating hormone by intraperitoneal (IP) injection (Section 3.2.4; Blake, 1997; Ertzeid & Storeng, 2001; Jackson Laboratories, 1988). A further 10-IU of chorionic gonadotropin was administered 48 hours later by IP injection. Superovulated recipient mice were immediately placed with vasectomised CBA/J male mice of proven sterility (unrestricted-coitus, Section 3.2.3). The following morning, superovulated recipient mice were checked for the presence of seminal plugs as per Section 3.2.3. If present, superovulated recipient mice were considered day-0.5 pc pseudo-pregnant (R0.5).

Immediately prior to ET, superovulated R2.5 recipient mice were anaesthetised using avertin anaesthetic (Section 3.2.11). Ten E3.5(-) embryos (five per uterine horn) were transferred into the uteri of each recipient mouse (80 embryos in total). The side of transfer (uterine horn) and selection of embryos were assigned using computer generated randomised lists to remove technician bias. Experimental design resulted in eight successful ET replicates (Figure 15) spread over two experimental series (two

weeks). One separate experimental series, which included three replicates, was excluded from the data set due to a lack of implants in all three recipient mice.

Seventeen days pc, recipient mice were euthanased and assessed as in Section 3.2.11. The numbers of viable and non-viable fetuses, and losses were recorded. Photos were taken of intact uteri, followed by the excised fetuses and their corresponding placentae. Each fetus and placenta was weighed, and gross morphology and development were assessed visually.

Standard deviation was used to indicate mouse-to-mouse variation. A number of statistical tests were used to confirm any significant differences between the assessed ET scenario and their non-superovulated counterpart. The numbers of implants, viable and non-viable fetuses and losses were analysed using Fisher's exact test. Fetal and placental weights were analysed using general linear modelling. Fetal:placental weight ratios were analysed using ANCOVA.

The asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice (Table 26, Section 4.4; Optimal model) was selected as the non-superovulated counterpart rather than natural mouse pregnancy. Two reasons led to this selection: 1) mean implantation rates were comparable if not better than natural mouse pregnancy; and 2) the asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice shared more common experimental design features with ET into SO recipient mice than natural mouse pregnancy. Thus, drew closer experimental comparison and helped minimise potential technical bias. It is also worth noting that this research did not focus on establishing models that closely reflected human implantation and IVF, but to gain an understanding of models that could use levels of implantation to measure IVF-intervention potential.
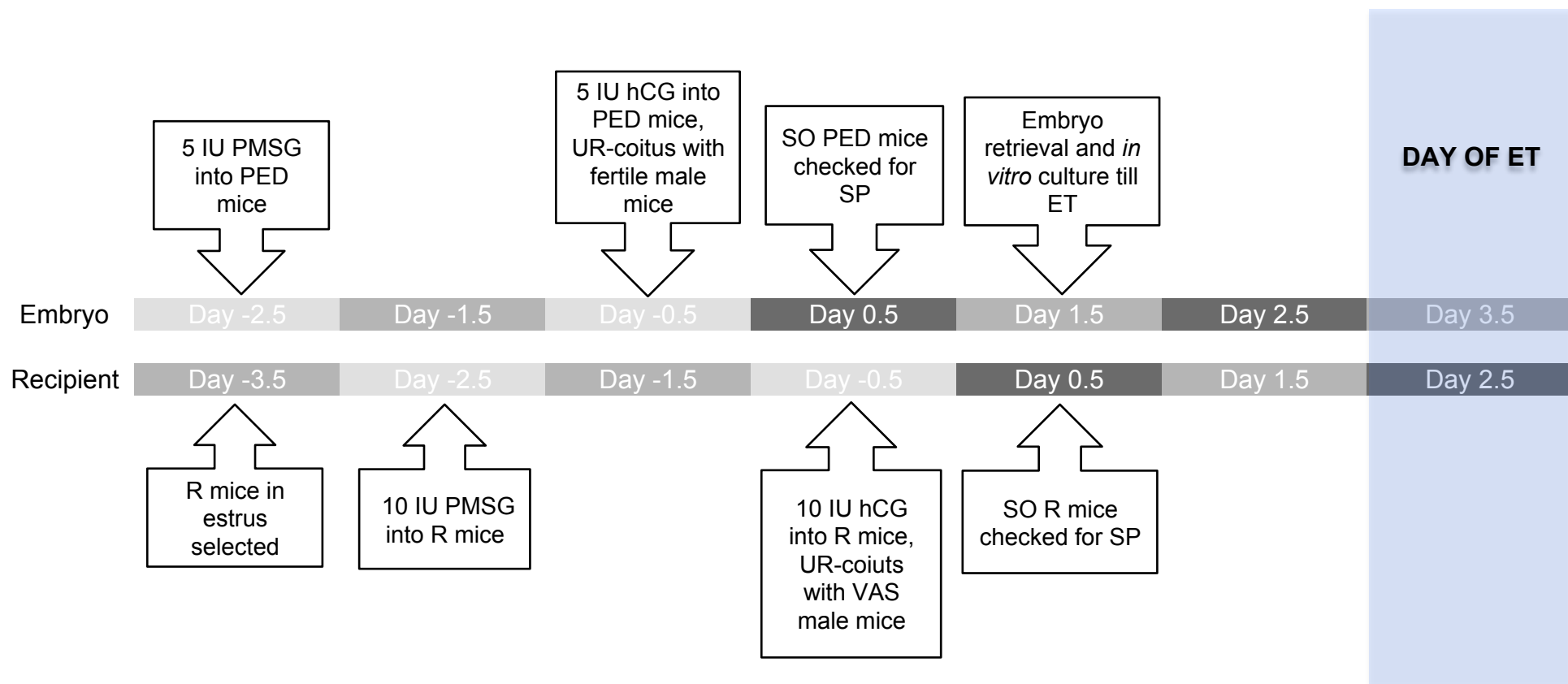
Figure 24 Schematic illustrating experimental design for ET into superovulated recipient mice. All superovulatory drugs (PMSG and hCG) were delivered by IP injection. Days are plus (+) or minus (–) of coitus. Key: IU, international units; PMSG, pregnant mare serum gonadotrophin; PED, pre-pubescent embryo donor; R, recipient; hCG, human chorionic gonadotrophin; UR, unrestricted; SO, superovulated; SP, seminal plug; VAS, vasectomised; ET, embryo transfer.

4.2.2   Results:

As shown in Table 21, the transfer of E3.5(-) embryos into superovulated R2.5(+) recipient mice resulted in a mean of 3.3 implants per recipient mouse (SD±4.5), which was significantly reduced compared to their non-superovulated counterpart (9.4, SD±1.1; $p < 0.001$). The impact of a standard deviation of 4.5 implants can be seen most clearly in Figure 25. Recipient mice 1 – 3 achieved an implantation rate of 8 - 9 implants per recipient mouse (Figure 25). In contrast, recipient mice 4 – 8 resulted in no implants. With the exception of one prematurely hatching embryo, grade allocation, all recipient mice received high quality embryos (Table 22). The small variation observed in stage of embryo development (M – 2A embryos), appeared to have little influence on implantation rates (Table 22). Weight of recipient mice did not influence numbers of implants per recipient mouse (Table 23).

Table 21 demonstrated that the mean number of viable fetuses (1.9, SD±2.8) was significantly decreased compared to their non-superovulated counterpart (6.9, SD±2.1; $p < 0.001$). Meanwhile, the reduction in mean numbers of non-viable fetuses (1.4, SD±2.1) demonstrated a strong tendancy towards significance (2.5, SD±1.8; $p = 0.055$). The remainder of embryos resulted in a loss (6.7, SD±4.5), which was significantly increased compared to non-superovulated recipient mice (0.5, SD±1.1; $p < 0.001$).

As shown in Table 21 and Figure 26, a significant decrease in the mean fetal weight (0.6 g, SD±0.1) was observed in comparison to those fetuses within non-superovulated recipient mice (1.0 g, SD±0.2; $p < 0.001$). In contrast, the mean placental weight (0.10 g, SD±0.02) was comparable to those of non-superovulated recipient mice (0.10 g, SD±0.02; $p = 0.462$). A mean fetal:placental weight ratio of 6.4 (SD±1.3) resulted, which was reduced compared to non-superovulated recipient mice (9.3, SD±1.3; $p = 0.015$). Gross morphology and development appeared normal, yet all fetuses were small for their gestational age, which reflected the low mean fetal:placental weight ratio (Figure 27).

Table 21 Consequences following transfer of E3.5(-) embryos (five per uterine horn) into eight superovulated R2.5(+) recipient mice. Sexually mature recipient mice (mean weight = 21.9 g) were stimulated with 10IU of PMSG and 10IU of hCG. Mean numbers of implants, viable fetuses, non-viable fetuses and losses; and the mean fetal and placental weights on day-17 pc. Mean numbers of implants, viable fetuses, mean fetal weights and fetal:placental ratios were significantly reduced compared to the Optimal Model ($p < 0.02$). In contrast, the mean numbers of losses were significantly increased ($p < 0.001$). Reduced mean numbers of non-viable fetuses demonstrated a strong tendancy towards significance ($p = 0.055$). All other outcomes were normal.

Outcome	Unit	ET into SO recipient mice		Optimal Model		P-value of means
		Mean per mouse (±SD)	Total	Mean per mouse (±SD)	Total	
Implants viable + non-viable fetuses	n	3.3 (±4.5)	26	9.4 (±1.1)	122	< 0.001[a]
Viable fetuses	n	1.9 (±2.8)	15	6.9 (±2.1)	90	< 0.001[a]
Non-viable fetuses	n	1.4 (±2.1)	11	2.5 (±1.8)	32	0.055[a]
Losses	n	6.7 (±4.5)	54	0.5 (±1.1)	6	< 0.001[a]
Fetal weight	g	0.6 (±0.1)	-	1.0 (±0.2)	-	< 0.001[a]
Placental weight	g	0.1 (±0.02)	-	0.1 (±0.02)	-	0.462
Fetal:placental weight ratio	-	6.4 (±1.3)	-	9.3 (±1.3)	-	0.015[a]

a = significantly different $p \leq 0.05$
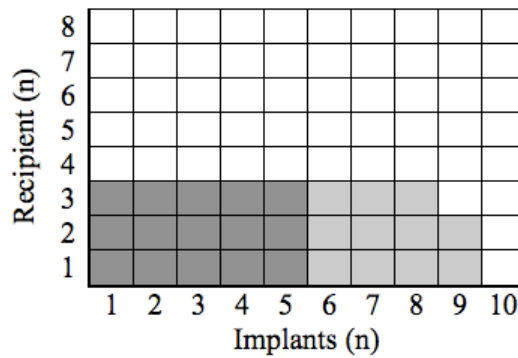
Figure 25 Impact of superovulated recipient mice on numbers and location of implants on day-17 pc following ET. Eight sexually mature recipient mice (mean weight = 21.9 g) were stimulated with 10IU of PMSG and 10IU of hCG, prior to ET. Features as per Figure 20. Recipient mice 4 – 8 resulted in no implants.

Table 22 Grade allocation of E3.5(-) embryos transferred into R2.5 SO recipient. Each recipient mouse represented one replicate, and was grouped as per their experimental series (Figure 15). Donor embryos were retrieved from a pool of prepubescent donor mice for each experimental series. Embryo grades, indicating stage of development and quality, were allocated as per Section 3.2.8 (Table 17). Mean numbers of implants were assessed on day-17 pc. Recipient 3 from Figure 25 represent Recipient 7 when grouped by experimental series. Key: Recip, recipient; Exp, experimental; ET, embryo transfer; M, morula; 5A - 2A, A grade blastocysts.

Recip	Exp series	Numbers per recipient mouse					
		Embryo grade at time of ET (%)					Implants
		M[a]	5A	4A	3A	2A	
1	1	10 (100)	-	-	-	-	9
2	1	6 (60)	-	4 (40)[b]	-	-	9
3	1	5 (50)	-	5 (50)	-	-	0
4	1	7 (70)	-	2 (20)	2 (20)	1 (10)	0
5	2	5 (50)	-	2 (20)	3 (30)	-	0
6	2	10 (100)	-	-	-	-	0
7	2	10 (100)	-	-	-	-	8
8	2	10 (100)	-	-	-	-	0

a = all morula A grade embryos
b = one 4A prematurely hatching, but cell integrity in tack.

Table 23 Weight of superovulated recipient mice pre-ET compared with delivery concentration of superovulatory drugs. Numbers of implants assessed day-17 pc. Recipient numbers corresponds with those identified in Figure 25.

Weight at time of ET (g)	SO drug concentration (IU / 10 g body weight)	Implants (n)	Recipient
20.2	4.9	0	7
20.3	4.9	9	2
20.4	4.9	8	3
20.5	4.9	9	1
22.4	4.5	0	4
23.2	4.3	0	8
23.9	4.2	0	5
24.2	4.1	0	6
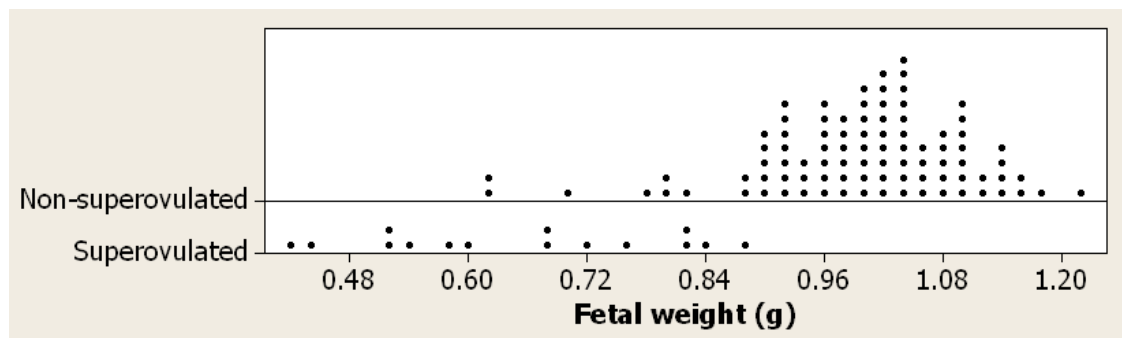
Figure 26 Impact of superovulated recipient mice on fetal weight following ET. Individual fetal weights on day-17 pc following the transfer of unmanipulated E3.5(-) embryos into superovulated or non-superovulated R2.5 recipient mice (Optimal Model). Superovulated recipient mice (mean weight = 21.9 g), were stimulated with 10IU of both PMSG and hCG. The reduction in fetal weight observed within superovulated recipient mice was significant (p < 0.001).

Left uterine horn   Right uterine horn

1cm
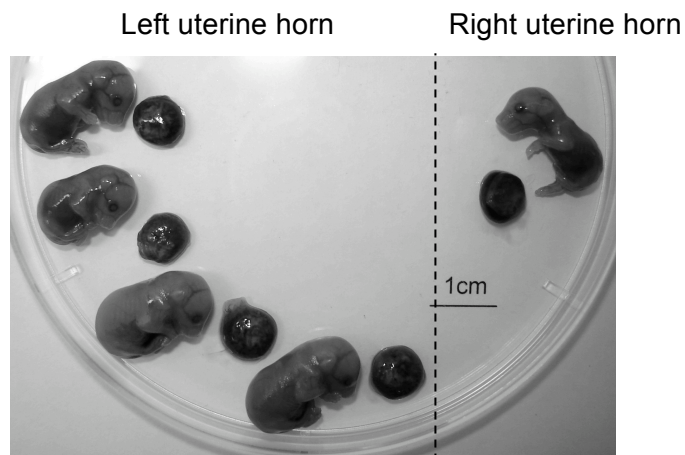
Figure 27 An example of gross morphology and development on day-17 pc within superovulated recipient mice following ET. Sexually mature recipient mice (mean weight = 21.9 g) were stimulated with 10IU of PMSG and 10IU of hCG, prior to ET. Position indicates location within uterine horn. Those placed at the top of the dish were closest to the oviduct. Those placed at the bottom of the dish were closest to the cervix. All fetuses were small for gestational age. Only three recipient mice resulted in viable fetuses, resulting in 3, 5 and 7 viable fetuses each.

4.2.3   Interpretation:

Following ET into superovulated recipient mice, the mean implantation rate was significantly reduced to 3.3 implants per recipient mouse (Figure 28). Comparatively, the mean numbers of losses were significantly increased (6.7). This indicated that the model prevented implantation. However, the mean implantation rate was substantially less than the specified 6 implants per recipient mouse. Not only that, but the mouse-to-mouse variability was substantially greater than ± 1 implant per recipient mouse (SD±4.5). Sixty-three percent of recipient mice did not demonstrate an implantation capability, while the remaining 37% demonstrate optimal implantation rates (8 - 9 implants per recipient mouse). Such variability was concerning, and raised questions surrounding technical issues.

It was difficult to justify the removal of all superovulated recipient mice that did not demonstrated implantation. A number of technical variables could have been responsible for this lack of implantation. These included: embryo quality, false positive pseudo-pregnancy, impact of superovulatory drugs in context of individual mouse weight, or a varied time available for embryo implantation-competency. Potential technical issues highlighted the importance of replicates and experimental series run over a number of weeks.

Technical issues surrounding embryo quality throughout this research were minimised in three ways. Firstly, the experimental design ensured that the embryo source was from a pool of donor embryo mice. Secondly, each recipient mouse had it's own set of mircrodrops and embryos. For this reason, the number of times embryos and their corresponding culture media were exposed to atmospheric conditions was standardized across replicates. Thirdly, if an entire series (one week) of replicates resulted in no implants, these recipient mice were removed from the data set. As indicated in Table 22, recipient mice received only high quality embryos.

The second potential variable that could have led to technical issues was a false positive observation of a seminal plug within superovulated recipient mice. This would lead to a false positive identification of pseudo-pregnancy within these superovulated recipient mice. In context of Sections 4.5 and 4.6, where manipulated and unmanipulated (media alone) embryos were transferred into the same recipient mouse, acting as it's own control, recipient mice were excluded from the data set when unmanipulated embryos did not implant. Out of the total number of ET, 20% of recipient mice were excluded in this way. Therefore, up to 20% of those superovulated recipient mice, which did not result in implants, could have been falsely identified as pseudo-pregnant. However, there was no way identifying this occurrence within superovulated recipient mice, and could not be used to exclude those mice from the data set. Nonetheless, Blake (1997) demonstrated a similar pregnancy rate (44%) to this research (38%). Thus, providing confidence that technical performance surrounding pseudo-pregnancy generation was comparable to the literature. In hindsight, it would have been useful to include the identification of CL just prior to ET, which was adopted later on in this research. Such a technique indicated that ovulation had taken place and that pseudo-pregnancy was possible. Consequently, the technique could have minimised the effects of false positive pseudo-pregnancies.

The impact of superovulatory drugs in context of individual mouse weight could have been responsible for the lack of implants. Within this research, each superovulated recipient mouse received a standard 10 IU per superovulatory drug, resulting in a mean delivery concentration of 4.6 IU / 10 g body weight. However, when individual weights were considered, delivery concentration varied from 4 – 5 IU / 10 g body weight. Nonetheless, Table 23 demonstrated that the small differences in superovulatory drug delivery concentration had no impact on the numbers of implants following ET.

The dramatic reduction in mean fetal weight (0.6 g) and a low mean fetal:placental weight ratio (6.4, SD±1.3) indicated poor intrauterine fetal growth and placental efficiency post-implantation (Barker, Bull, Osmond, & Simmonds, 1990; Mu, Slevin, Qu, McCormick & Adamson, 2008). While this outcome reflects birth weights following human IVF (fresh embryo transfer) the focus of this research was not to establish models that closely reflected human implantation and IVF (Kalra, Ratcliffe, Coutifaris, Molinaro, & Barnhard, 2011). Research focused on levels of implantation that were useful to measure IVF-intervention potential. In other words, level of implantation was a tool, which could measure degrees of success. Therefore, reduced mean fetal weight carried limited influence over the selection of the most suitable suboptimal model. Overall data indicated that while ET into SO recipient mice did prevent implantation, mean numbers of implants were substantially lower than the ideal 6 implants per recipient mouse (±1 implant). Consequently, the model would not be assessed for its potential to detect positive consequences of manipulated embryos.
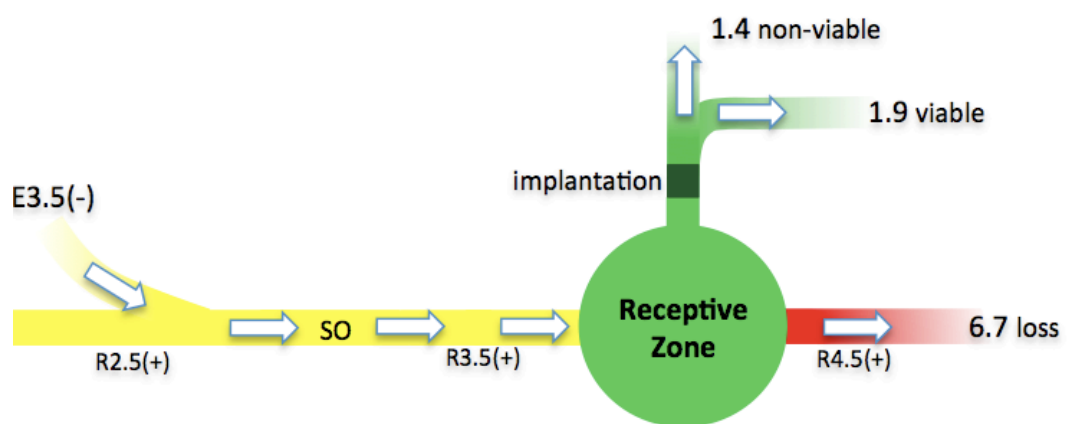
Figure 28 Schematic representation of the impact of superovulated recipient mice on ET mean outcomes. Prior to ET, eight sexually mature recipient mice (mean weight = 21.9 g) were stimulated with 10IU of PMSG and 10IU of hCG. For every 10 E3.5(-) embryos transferred into superovulated R2.5 recipient mice, a mean of 3.3 successfully implanted. A mean of 1.9 led to viable fetuses and 1.4 led to non-viable fetuses. The remaining embryos failed to implant prior to end of the receptive zone, leading to a mean of 6.7 losses.

4.3 Administration of progesterone antagonist following ET

Administration of progesterone antagonists, such as RU486, can elicit an abortive, contragestive or contraceptive effect *in utero*. The mechanism of action is time and dose dependent. Within this research, RU486 was subcutaneously administered one-day post-ET (approximately 87 hours pc). At the time of experimentation, endometrial receptivity, thus implantation, was thought to begin by 92 hours pc (Finn & McLaren, 1967; McMaster, et al., 1993). Therefore, in the context of the literature, RU486 was administered just prior to implantation. Such timing and dose, specified in Section 3.2.10, meant RU486 would have a contragestive effect, which could act through a number of mechanisms. These could include: inhibited endometrial mitoses; delayed pinopode development; reduced embryo apposition or invasion (Gao, et al., 2001; Huang, et al., 2005; Li, et al., 2005; Liu, et al., 2003; Liu, et al., 2008; Sarantis, et al., 1998; Table 18, Section 3.2.9). As a result, only a proportion of the endometrium would become receptive, creating less implantation opportunity (Figure 29). Inhibited blastocyst development and viability were unlikely, as donor embryos were produced and cultured within an environment lacking RU486 (Lalitkumar, et al., 2007). In addition, blastocyst development and implantation-competency would have been close to completion by the time of RU486 administration.

While experimental design suggested RU486 would have a contragestive effect, reduced embryo invasion could lead to an abortive effect post-implantation (increased non-viable fetuses; "Antiprogestogens: from abortion to contraception," 1992). For this reason, it was necessary to confirm that the model's action prevented implantation and was able to achieve a suboptimal implantation rate.
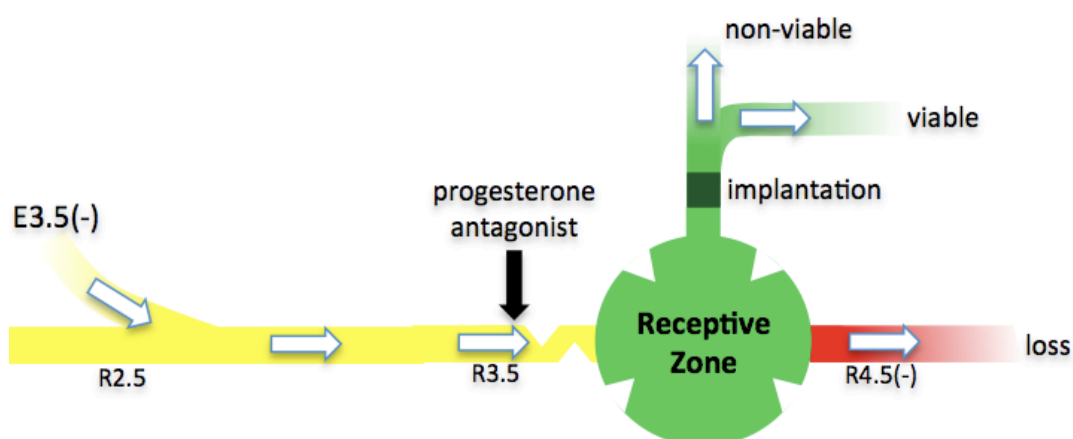
Figure 29 Schematic representation of progesterone antagonist impact on endometrial maturation. Progesterone antagonists, in the form of RU486, were administered one day following ET (R3.5). A proportion of endometrial cells slow in their development (missing portions of schematic pathway). Thus, resulting in less receptive endometrium available to the fully developed embryo. The negative symbol (-) indicated delayed embryo and endometrial development compared real-time.

4.3.1   Methodology overview:

As per Section 3.2.7, pre-pubescent donor B6CBAF1/J mice were stimulated with superovulatory drugs and embryos retrieved on day-1.5 pc (Figure 30). Embryos were then *in vitro* cultured in 5% CO_2 at 37°C for two days. Pseudo-pregnant B6CBAF1/J recipient mice were produced by restricted-coitus with vasectomised CBA/J male mice of proven sterility (Sections 3.2.2 and 3.2.3). Restricted-coitus was enabled by specialised cages that limited the length of time vasectomised male mice had access to recipient mice (Section 3.2.3; Figure 14). As a result, the majority of recipient mice would undergo coitus at approximately the same time. Therefore, the stage of endometrial maturation at any one time would be similar between recipient mice.

Immediately prior to ET, R2.5 recipient mice were anaesthetised using avertin anaesthetic (Section 3.2.9). As per the Optimal model (Section 4.4), 10 E3.5(-) embryos (five per uterine horn) were transferred into the uteri of each recipient mouse (100 embryos in total). The side of transfer (uterine horn) and selection of embryos were assigned using computer generated randomised lists to remove technician bias. Experimental design resulted in ten successful ET replicates Figure 15 spread over three experimental series (three weeks).
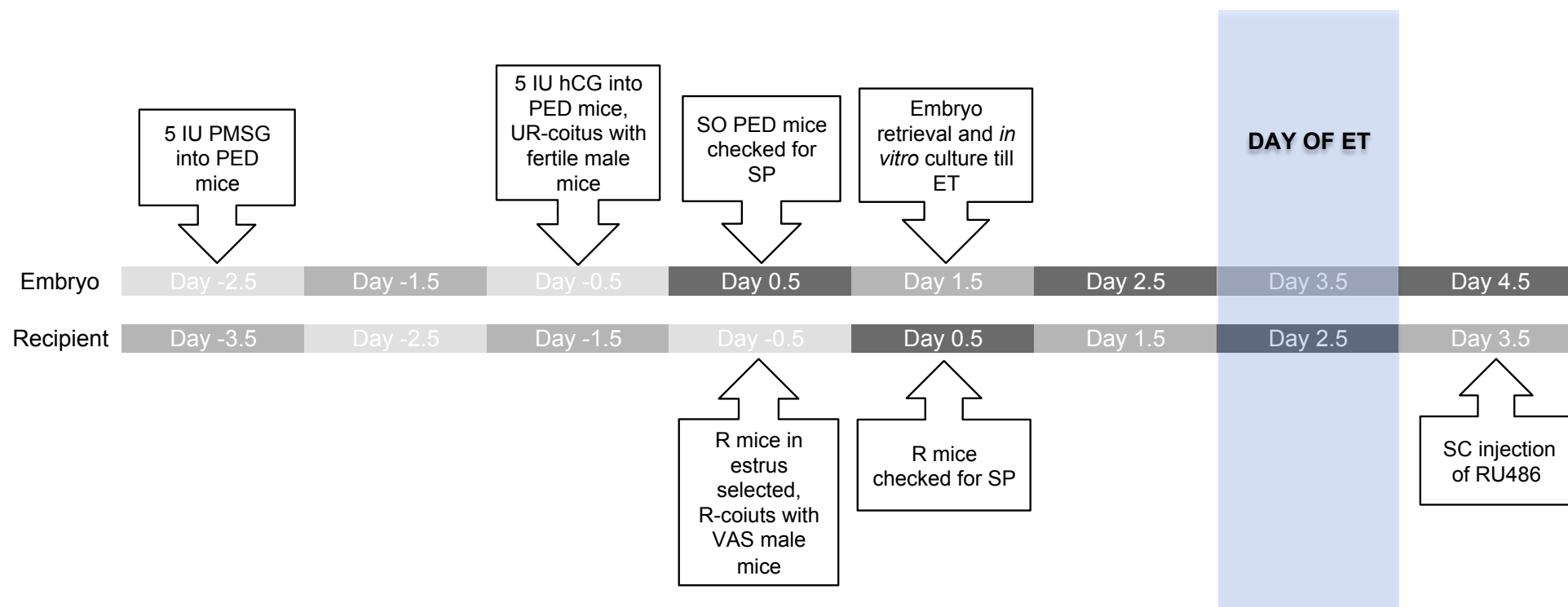
Figure 30 Schematic illustrating experimental design for progesterone antagonist administration following ET. Days are plus (+) or minus (–) of coitus. One day following the transfer of 10 E3.5(-) embryos into R2.5 recipient mice, RU486, a progesterone antagonist, was injected subcutaneously at 2.0 x10^{-5} g / 10 g of body weight. All other features as per Figure 24. Key: R, restricted; SC, subcutaneous injection.

One day post-ET, recipient mice were injected subcutaneously into the scruff of the neck with RU486, a progesterone antagonist, at 2.0×10^{-5} g / 10 g of body weight (Section 3.2.10). Note that 3.0×10^{-5} g / 10 g of body weight was also trialled resulting in nearly zero implantation (Table 68). Timing and dose of administration was chosen in reflection of Goa, et al. (2001) and Liu, et al., (2003; 2008). Seventeen days pc, recipient mice were euthanased and ET outcomes assessed (Section 3.2.11). The numbers of viable and non-viable fetuses, and losses were recorded. Photos were taken of intact uteri, followed by the excised fetuses and their corresponding placentae. Each fetus and placenta was weighed, and gross morphology and development were assessed visually. At the time of experimentation, which was early on in the PhD journey, data collated from the Optimal model was used as control. In hindsight, a second control, where sham injections of propylene glycol, the diluent used for RU486, would have been beneficial. Statistical analysis was performed as in Section 4.2.

4.3.2 Results:

Following the administration of progesterone antagonist, the mean number of implants (3.1, SD±4.0) was significantly reduced in comparison to those recipient mice that did not receive such a drug (9.4, SD±1.1; $p < 0.001$; Table 24). Figure 31 demonstrates the substantial variation seen mouse-to-mouse, where at least 50% of recipient mice did not result in implants. While recipient mice 1 – 5, resulted in 1 – 10 implants per recipient mouse, recipient mice 6 – 10 resulted in no implants (Figure 31). As demonstrated in Table 25, all recipient mice received high quality embryos. The small variation observed in stage of embryo development (M - 4A embryos), appeared to have little influence on implantation rates (Table 22).

Of those embryos that successfully implanted, a mean of 1.7 (SD±2.4) resulted in a viable fetus and 1.4 (SD±2.3) in a non-viable fetus (Table 24). Both values were significantly decreased compared to those recipient mice that did not receive any progesterone antagonist (6.9, SD±2.1; 2.5, SD±1.8; $p < 0.05$). A significant increase was seen in the number losses (6.9, SD±4.0) compared to those lost within recipient mice devoid of any progesterone antagonist (0.5, SD±1.1; $p < 0.001$).

The mean fetal weight (0.9 g, SD±0.2) was significantly reduced compared to recipient mice that had not received any progesterone antagonist (1.0 g, SD±0.2; $p = 0.001$; Figure 32). In contrast, the mean placental weight (0.13 g, SD±0.03) was significantly increased (0.1 g, SD±0.02; $p < 0.001$; Figure 33). A mean fetal:placental weight ratio

of 6.5 (SD±1.6) resulted, which was reduced compared to the Optimal model (9.3, SD±1.3; $p < 0.001$).   Normal gross morphology and development were observed (Figure 34). Although sham injections of propylene glycol were not delivered as a second control, recipient mouse health remained normal following the administration of RU486.

Table 24 Consequences of progesterone antagonist administration into the Optimal Model one-day post-transfer. Progesterone antagonist (RU486) subcutaneously administered into ten recipient mice, at 2.0 x10^{-5} g / 10 g of body weight, one-day post-transfer. Mean numbers of implants, viable fetuses, non-viable fetuses and losses, and the mean fetal and placental weights were assessed on day-17 pc. Mean numbers of implants, viable and non-viable fetuses, mean fetal weight and fetal:placental ratios were significantly reduced (p < 0.001). In contrast, mean numbers of losses, and the mean placental weight were significantly increased (p < 0.001).

Outcome	Unit	Progesterone antagonist administration one-day post-ET		Optimal Model		P-value of means
		Mean per mouse (±SD)	Total	Mean per mouse (±SD)	Total	
Implants viable + non-viable fetuses	n	3.1 (±4.0)	31	9.4 (±1.1)	122	< 0.001[a]
Viable fetuses	n	1.7 (±2.4)	17	6.9 (±2.1)	90	< 0.001[a]
Non-viable fetuses	n	1.4 (±2.3)	14	2.5 (±1.8)	32	0.046[a]
Losses	n	6.9 (±4.0)	69	0.5 (±1.1)	6	< 0.001[a]
Fetal weight	g	0.9 (±0.2)	-	1.0 (±0.2)	-	0.001[a]
Placental weight	g	0.13 (±0.03)	-	0.1 (±0.02)	-	< 0.001[a]
Fetal:placental weight ratio	-	6.5 (±1.6)	-	9.3 (±1.3)	-	< 0.001[a]

a = significantly different p ≤0.05
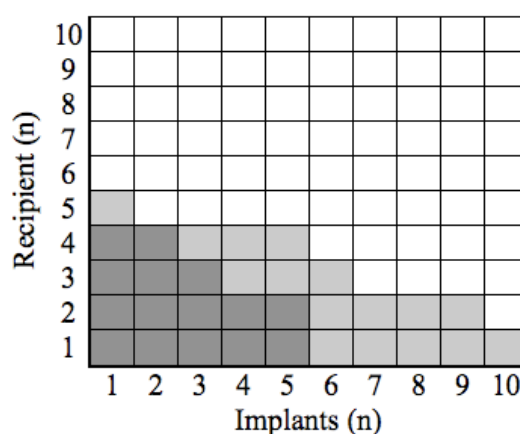
Figure 31 Impact of progesterone antagonist administration on the numbers and location of implants within the Optimal Model on day-17 pc. Progesterone antagonist (RU486) subcutaneously administered into ten recipient mice, at 2.0 x10^{-5} g / 10 g of body weight, one-day post-transfer. Features as per Figure 20. Recipient mice 6 - 10 resulted in no implants.

Table 25 Grade allocation of E3.5(-) embryos transferred into R2.5 recipient mice prior to progesterone antagonist administration. Each recipient mouse represented one replicate, and was grouped by experimental series (Figure 15). Donor embryos were retrieved from a pool of prepubescent donor mice for each experimental series. Embryo grades, indicating stage of development and quality, were allocated as per Section 3.2.8 (Table 17). Mean numbers of implants were assessed on day-17 pc. Recipients 1, 3, 5, 7 and 9 represent recipients 3, 5, 2, 4 and 1 in Figure 31. Key: Recip, recipient; Exp, experimental; ET, embryo transfer; M, morula; 5A - 2A, A grade blastocysts.

Recip	Exp series	Numbers per recipient mouse			
		Embryo grade at time of ET (%)			Implants
		M[a]	5A	4A	
1	1	9 (90)	-	1 (10)	6
2	1	10 (100)	-	-	0
3	1	10 (100)	-	-	1
4	1	10 (100)	-	-	0
5	2	10 (100)	-	-	9
6	2	8 (80)	-	2 (20)	0
7	2	10 (100)	-	-	5
8	2	10 (100)	-	-	0
9	2	10 (100)	-	-	10
10	3	10 (100)	-	-	0

a = all morula A grade embryos
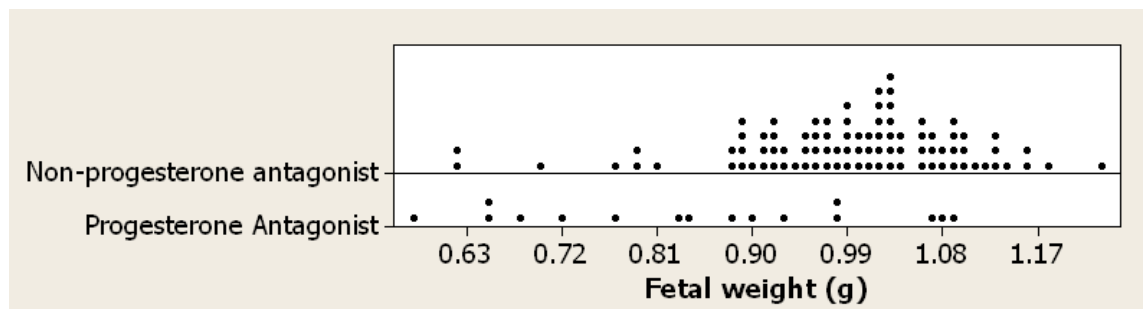
Figure 32 Impact of progesterone antagonist administration on fetal weight day-17 pc. Progesterone antagonist (RU486) subcutaneously administered into ten recipient mice, at 2.0 x10^{-5} g / 10 g of body weight, one-day post-transfer. Data was compared with recipient mice that did not receive any progesterone antagonist (Optimal Model). Significantly reduced mean fetal weight was observed (p < 0.001).
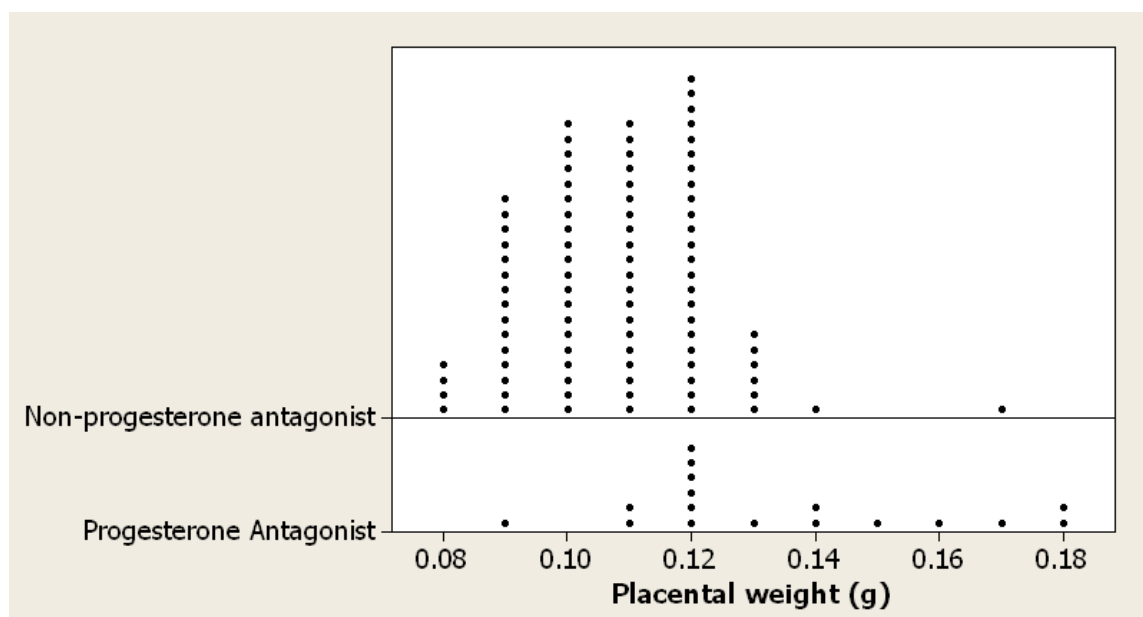
Figure 33 Impact of progesterone antagonist administration on placental weights day-17 pc. Ten recipient mice subcutaneously received progesterone antagonist (RU486), at 2.0 x10^{-5} g / 10 g of body weight, one-day post-transfer. Recipient mice that did not receive any progesterone antagonist were used as a control (Optimal Model). Significantly increased mean placental weights were observed (p < 0.001).

116

Left uterine horn   Right uterine horn
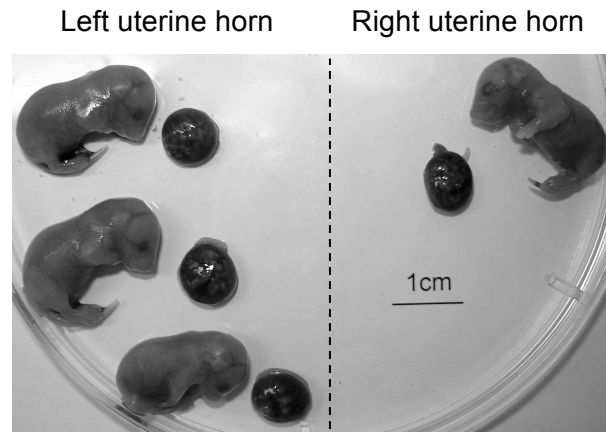
Figure 34 An example of gross morphology and development day-17 pc following progesterone antagonist administration within the Optimal Model one-day post-transfer. Layout as per Figure 59. Progesterone antagonist (RU486) subcutaneously administered into ten recipient mice, at 2.0×10^{-5} g / 10 g of body weight, one-day post-transfer. Five recipient mice resulted in viable fetuses, ranging from 1 – 7 viable fetuses each. Fetus located in bottom left hand corner was slightly small for gestational age (0.84 g). Corresponding placenta (0.14 g) and the placenta located directly above (0.15 g) were large for gestational age. All other fetuses and placentae normal for gestational age.

4.3.3   Interpretation:

The administration of progesterone antagonist, into the Optimal Model only met one of the two selection criteria. A reduced mean implantation rate (3.1) and an increased mean numbers of losses (6.9) confirmed that the model prevented implantation (first criterion). However, not only did the mean implantation rate fall substantial short of 6 implants per recipient mouse, but it also exceeded acceptable levels of variation (± 1 implant; second criterion).
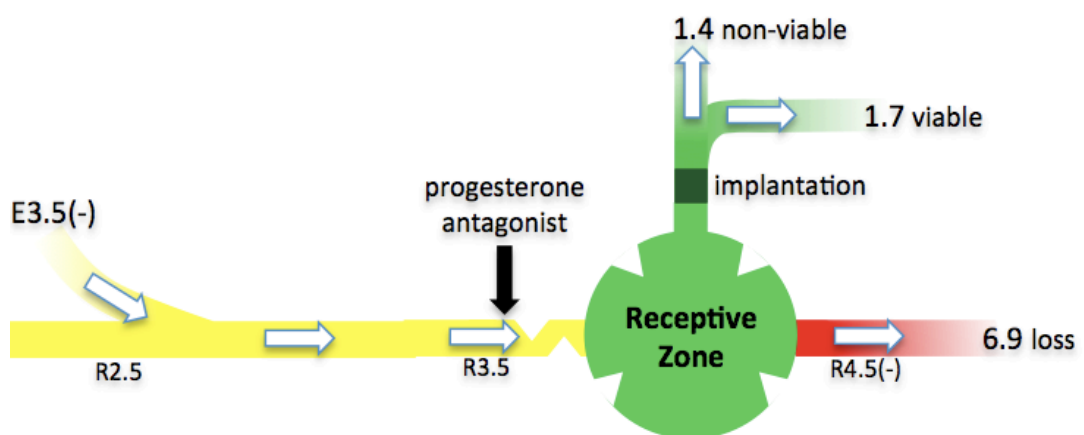
Figure 35 Schematic representation of the impact of progesterone antagonist administration on ET mean outcomes. Progesterone antagonist (RU486) subcutaneously administered into ten recipient mice, at 2.0×10^{-5} g / 10 g of body weight, one-day post-transfer. For every 10 E3.5(-) embryos transferred into R2.5 recipient mice, a mean of 3.1 successfully implanted. A mean of 1.7 led to viable fetuses and 1.4 led to non-viable fetuses. The remaining

embryos failed to implant prior to end of the receptive zone, leading to a mean of 6.9 losses.

Individual data clearly demonstrated the excessive variability. Firstly, 50% of recipient mice did not demonstrate an implantation capability. Secondly, the remaining recipient mice demonstrated suboptimal (1.0 implants) through to the maximum possible implantation rates (10.0 implants). Thirdly, at least 40% of these recipient mice achieved optimal implantation rates. Such variability was concerning and raised questions regarding the consistency of the model. If manipulated embryos were transferred into such a model, any subtle positive consequences could be missed.

While the mean fetal weight (0.9 g) was reduced by only 0.1 g compared to the Optimal Model (1.0 g), the difference was significant (p = 0.001). Yet, 50% of the individual fetal weights resided within normal weight ranges. A similar pattern was observed regarding placental weights. The mean placental weight (0.13 g) was significantly higher than in the Optimal Model (0.12 g; $p < 0.001$), yet 50% of individual weights resided within normal placental weight ranges. Those placental weights that were high led to a mean fetal:placental weight ratio of 6.4 (SD±1.3), which would normally indicate poor placental proficiency. It could be said that while fetal weights were close to normal, in context of the increased placental weights, fetal weight was small. Although, this data were associated with post-implantation events, it supported concern regarding the model's consistency and potential mechanisms of action outside of delayed endometrial development. Potential mechanisms of action included: reduced expression of Fas and FasL genes, thus reducing apposition at the implantation site; and reduced embryo invasion. Consequently, the model generated by progesterone antagonist administration would not be assessed for its potential to detect positive consequences of manipulated embryos.

4.4 Synchronous and asynchronous ET

Within this section, synchronous and asynchronous ET scenarios were assessed for their ability to generate optimal and suboptimal implantation rates. Within these models, the developmental age of donor embryos and recipient endometrial was simply shifted at the time of transfer. As a consequence, synchronous and asynchronous ETs were considered far simpler to establish compared to ET into SO recipient mice or progesterone antagonist administration following ET (Sections 4.2 and 4.3).

Embryo transfers were considered synchronous when the developmental age, in days pc, of donor embryos and recipient mouse endometrium were equal (Figure 36). Their developmental age was measured from the time of coitus, which approximately took place at the midpoint of the dark phase immediately prior to the morning of day-0.5 pc.   *In vivo* implantation murine models that were generated using synchronous ET were analogous to human and animal ET.   In contrast, an ET was considered asynchronous when the developmental age of donor embryos, in days-pc, was more or less than recipient mouse endometrium. This form of ET generated an *in vivo* implantation murine model partially analogous to frozen human IVF cycles, which included natural and stimulated cycles (Figure 37).   While embryo-endometrial developmental synchrony is unlikely to be out by more than one day in the clinical context, ET within pigs or sheep can be up to two days asynchronous (Webel, et al., 1970; Wilmut, et al., 1988; Wilmut & Sales, 1981).
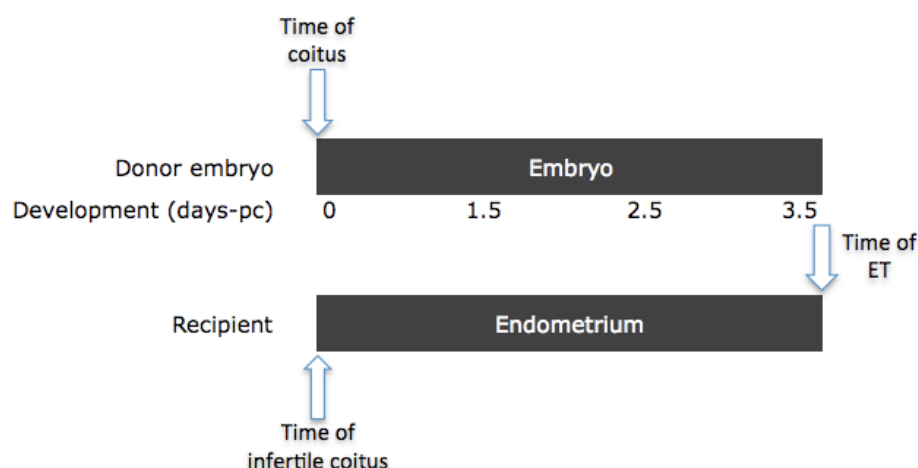
Donor embryo development equal to recipient mouse endometrium

Figure 36 Schematic representation of synchronous ET. The length of time between coitus and ET for both donor embryos and recipient mice is the same number of days.

Within Section 4.4, four different ETs scenarios have been assessed (Figure 38). They included: 1) the asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice; 2) the asynchronous transfer of E4.5(-) embryos into R2.5 recipient mice; 3) the synchronous transfer of E3.5(-) embryos into R3.5 recipient mice; and 4) the

asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice. At this point, it is important to note that the transfer of E3.5(-) embryos into R3.5 recipient mice was not strictly synchronous. While both donor embryos and recipient mouse endometrium shared the same real-time developmental age (3.5-days pc), their stage of development could differ. This potential lack of synchrony was attributed to the use of *in vitro* verse *in vivo* cultured superovulated donor embryos. Embryos that have been cultured *in vitro* are known to exhibit delayed development compared to their natural counterparts (Edwards, et al., 2005; Ertzeid & Storeng, 1992, 2001; Van der Auwera & D'Hooghe, 2001). Some features include: reduced cleavage rates and blastocyst formation; and altered gene expression and metabolic activity (Bowman & McLaren, 1970; Fleming, et al., 2004; Harlow & Quinn, 1982; Rinaudo & Schultz, 2004; Watkins, et al., 2007). Selection of culture media and conditions are a major influencing factor. Nevertheless, the experimental design utilised was widely accepted throughout literature, and ensured high quantity of embryos, while still maintaining quality analogous to natural mouse pregnancy (Hogan, et al., 1994). Therefore, any potential influence a lack of synchrony held on resulting data was considered minimal.

Findings associated with each ET scenario would be used to select the most suitable optimal and suboptimal models. In addition to the two criteria associated with models that achieved low levels of implantation, specified in Section 4.2, a further two criteria would be used to assess models that achieved high levels of implantation. These included: 1) for every 10 embryos transferred, the mean implantation rate would be comparable or greater than natural mouse pregnancy (9.0 implants per mouse ± 1 implant); and 2) at least 70% of implants would need to result in a viable fetus. The first criterion ensured that the Optimal Model did not have any adverse effects on implantation. Furthermore, any negative consequences following the transfer of manipulated embryos would be clearly observed by a reduction in the mean implantation rate. The second criterion ensured that numbers of experimental mice were kept to a minimum. Such an optimal model would have sufficient numbers of viable fetuses to measure fetal and placental weights, and to assess gross morphology and development. The most suitable optimal and suboptimal models have been carried through to Sections 4.5 and 4.6, where their potential to detect consequences of manipulated embryos are assessed.
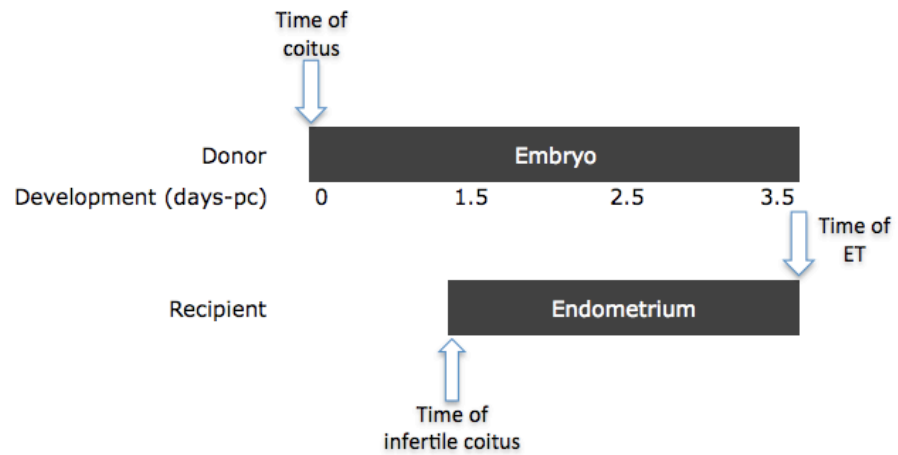
Schematic A – Donor embryo development more advanced than recipient mouse endometrium
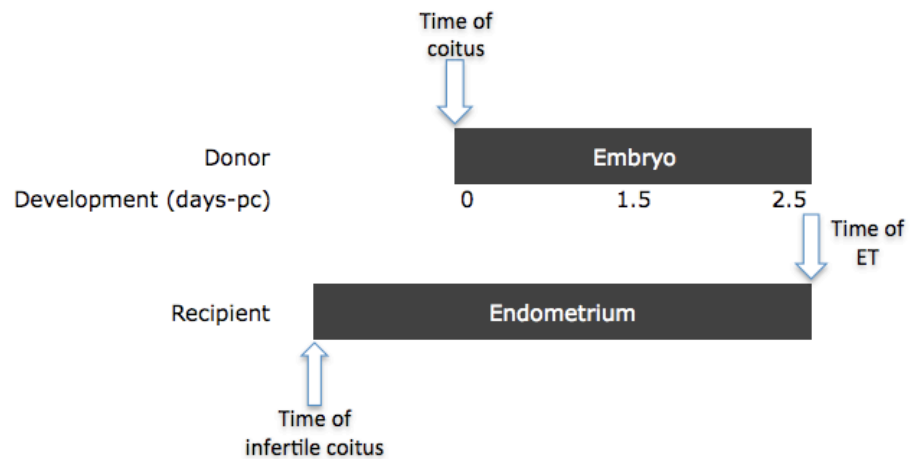
Schematic B – Donor embryo development less advanced than recipient mouse endometrium

Figure 37 A schematic representation of asynchronous ET. Asynchronous ETs are generated when the extent donor embryo development is more (Schematic-A) or less (Schematic-B) days than the recipient mouse endometrium.
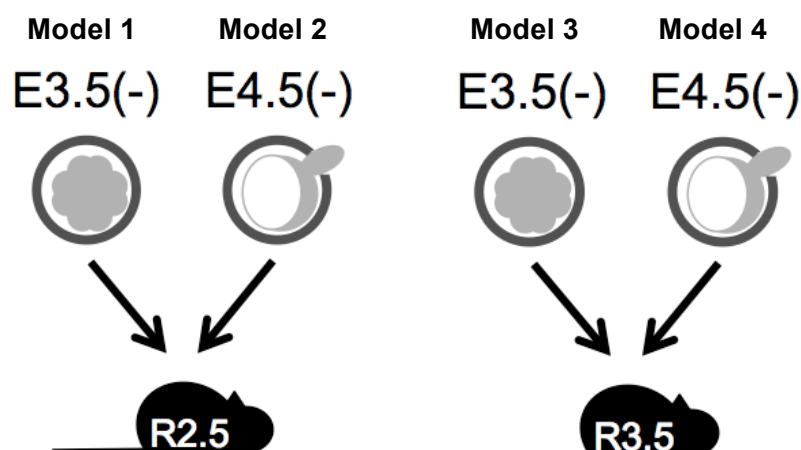
Figure 38 Schematic drawings illustrating four different unmanipulated *in vivo* implantation murine models. Asynchronous transfer of E3.5(-) embryos (morula) or E4.5(-) embryos (hatching blastocysts) into R2.5 recipient mice created the first and second models. Synchronous transfer of E3.5(-) embryos into R3.5 recipient mice generated the third model. Asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice created the fourth and final model.

4.4.1   Methodology overview:

Production and collection of embryos from pre-pubescent donor B6CBAF1/J mice on day-1.5 pc, was performed as per Section 3.2.7. Embryos were then *in vitro* cultured in 5% CO_2 at 37°C until days-3.5 or 4.5 pc. Pseudo-pregnant B6CBAF1/J recipient mice were produced by restricted-coitus with vasectomised CBA/J male mice of proven sterility (Sections 3.2.2 and 3.2.3). However, the experiments investigating the asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice were completed prior to the introduction of specialized cages in Section 4.3, which allowed for restricted coitus. For this reason unrestricted-coitus was used to generate R2.5 recipient mice that received E3.5(-) embryos.

Ten embryos (five per uterine horn) were transferred into the uteri of pseudo-pregnant recipient mice as per Figure 38, and Section 3.2.9. Embryo transfer outcomes were assessed on day-17 pc (Section 3.2.11). The numbers of viable and non-viable fetuses, and losses were recorded. Photos were taken of intact uteri, followed by the excised fetuses and their corresponding placentae. Each fetus and placenta was weighed, and gross morphology and development were assessed visually.

Standard deviation was used to indicate mouse-to-mouse variation. A number of statistical tests were used to confirm any significant differences between the assessed each ET scenario and natural mouse pregnancy. The numbers of implants, viable and non-viable fetuses and losses were analysed using Fisher's exact test. Fetal and placental weights were analysed using general linear modelling. Fetal:placental weight ratios were analysed using ANCOVA.

4.4.2   Results:

4.4.2.1   Asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice

Figure 39 summarises the experimental design leading up to the asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice. Thirteen replicates were completed over six experimental series (six weeks; Figure 15).

Data in Table 26 demonstrated that the transfer of *in vitro* cultured E3.5(-) embryos into R2.5 recipient mice produced a mean implantation rate of 9.4 implants per recipient mouse (SD±1.1). The small increase in mean implantation rate compared to natural mouse pregnancy (9.0, SD±0.9) demonstrated a strong tendancy towards significance (p = 0.084). Implantation rates within individual recipient mice ranged from 7 – 10 implants per recipient mouse (Figure 40). A maximum implantation rate of 10 implants per recipient mouse was achieved in 70% of recipient mice. The majority of recipients received A-graded embryos (Table 27). Although some recipient mice received B-graded embryos, maximum numbers of implants were still achieved within these mice.

Of those embryos that implanted, a mean of 6.9 (SD±2.1) resulted in viable fetuses and 2.5 (SD±1.8) in non-viable fetuses (Table 26). Correspondingly, the mean number of viable fetuses was significantly reduced compared to natural mouse pregnancy (8.7, SD±1.2; p < 0.001), the mean number of non-viable fetuses was significantly increased (0.3, SD±0.7; p < 0.001). While the mean numbers of embryos that resulted in a loss (0.5, SD±1.1) were comparable to natural pregnancy (0.0), the small increase demonstrated a strong tendacy towards significance (p = 0.084).

The mean fetal (1.0 g, SD±0.2) and placental (0.1 g, SD±0.02) weights were comparable to natural mouse pregnancy (1.0 g, SD±0.07; 0.1 g, SD±0.01; p ≥ 0.145). The resulting mean fetal:placental ratio (9.3, SD±1.3) was significantly increased compared to natural mouse pregnancy (8.5, SD±1.0; p < 0.001). Gross morphology and development were also comparable to natural mouse pregnancy.

Figure 39 Schematic summarising the experimental design for the asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice. Days are plus (+) or minus (–) of coitus. All other features as per Figure 24.

Table 26 Asynchronous transfer of E3.5(-) embryos (five per uterine horn) into 13 R2.5 recipient mice mean outcomes. Mean numbers of implants, viable fetuses, non-viable fetuses and losses day-17 pc. Mean numbers of viable fetuses were significantly decreased, while non-viable fetuses and their fetal:placental ratios were significantly increased compared to natural pregnancy (p < 0.001). All other outcomes were normal.

Outcome	Unit	E3.5(-) / R2.5		Natural pregnancy		P-value of means
		Mean per mouse (±SD)	Total	Mean per mouse (±SD)	Total	
Implants viable + non-viable fetuses	n	9.4 (±1.1)	122	9.0 (±0.9)	81	0.084
Viable fetuses	n	6.9 (±2.1)	90	8.7 (±1.2)	78	< 0.001[a]
Non-viable fetuses	n	2.5 (±1.8)	32	0.3 (±0.7)	3	< 0.001[a]
Losses	n	0.5 (±1.1)	6	0.0	0	0.084
Fetal weight	g	1.0 (±0.2)	-	1.0 (±0.07)	-	0.285
Placental weight	g	0.1 (±0.02)	-	0.12 (±0.01)	-	0.145
Fetal:placental weight ratio	-	9.3 (±1.3)	-	8.5 (±1.0)	-	< 0.001[a]

a = significantly different p ≤0.05

Table 27 Grade allocation of E3.5(-) embryos asynchronously transferred into R2.5 recipient mice. Each recipient mouse represented one replicate, and was grouped by experimental series (Figure 15). Donor embryos were retrieved from a pool of prepubescent donor mice for each experimental series. Embryo grades, indicating stage of development and quality, were allocated as per Section 3.2.8 (Table 17). Mean numbers of implants were assessed on day-17 pc. Recipients 1, 7, 9, and 12 represent recipients 12, 11, 10 and 13 in Figure 40. Key: Recip, recipient; Exp, experimental; ET, embryo transfer; M, morula; 5A - 2A, A grade blastocysts.

Recip	Exp series	Numbers per recipient mouse					Implants
		Embryo grade at time of ET (%)					
		M[a]	5A	4A	3A	2A	
1	1	10 (100)	-	-	-	-	7
2	1	10 (100)	-	-	-	-	10
3	1	8 (80)	-	2 (20)	-	-	10
4	2	10 (100)	-	-	-	-	10
5	2	4 (40)	-	6 (60)[b]	-	-	10
6	3	9 (90)	-	1 (10)[c]	-	-	10
7	3	10 (100)	-	-	-	-	9
8	4	10 (100)	-	-	-	-	10
9	5	8 (80)	-	1 (10)	1 (10)	-	9
10	5	10 (100)	-	-	-	-	10
11	6	10 (100)	-	-	-	-	10
12	6	10 (100)	-	-	-	-	7
13	6	4 (40)	-	3 (30)	2 (20)	1 (10)	10

a = all morula A grade embryos
b = four 4B embryos
c = B grade.
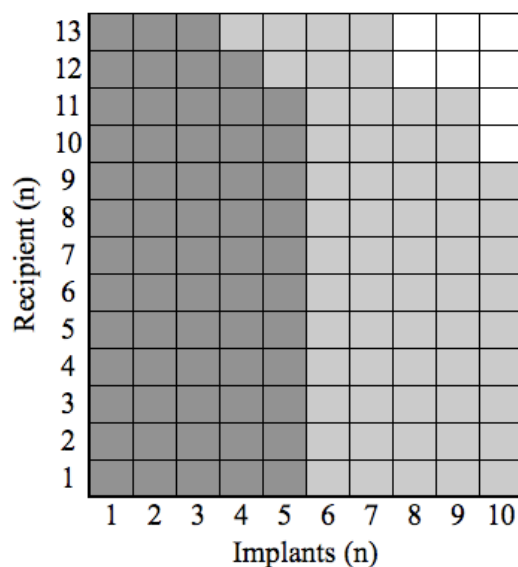
Figure 40 Numbers and location of implants within following the asynchronous transfer of E3.5(-) embryos (five per uterine horn) into 13 R2.5 recipient mice. Features as per Figure 20. Recipient mice 1 – 9 resulted in maximum numbers of implants. Mean implantation rate was 9.4 implants per recipient mouse (SD±1.1).

4.4.2.2 Asynchronous transfer of E4.5(-) embryos into R2.5 recipient mice

The experimental design summary, leading up to the asynchronous transfer of E4.5(-) embryos into R2.5 recipient mice, can be seen in Figure 41. Eight replicates (recipient mice) were completed over two experimental series (two weeks; Figure 15).

As seen in Table 28, the transfer of E4.5(-) embryos into R2.5 recipient mice resulted in a mean implantation rate of 9.8 implants per recipient mouse (SD±0.5), which was comparable to natural mouse pregnancy (9.0, SD±0.9; p = 0.713). Figure 42 demonstrates the consistency of the model, where 75% of recipient mice resulted in the maximum possible number of implants (10.0). The remaining 25% resulted in 9.0 implants per recipient mouse. All but one transferred embryo were A-grade quality, yet, maximum numbers of implants were achieved within that recipient mouse (Table 29).

Figure 41 Schematic summarising the experimental design for the asynchronous transfer of E4.5(-) embryos into R2.5 recipient mice. Days are plus (+) or minus (–) of coitus. All other features as per Figure 24. Key: R, restricted.

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Table 28 Mean outcomes of asynchronous transfer of E4.5(-) embryos (five per uterine horn) into eight R2.5 recipient mice. Mean numbers of implants, viable fetuses, non-viable fetuses and losses; and the mean fetal and placental weights day-17 pc. Mean numbers of viable fetuses were significantly decreased, while non-viable fetuses were significantly increased (p < 0.03). The increase in mean fetal:placental ratio had a strong tendancy towards significancy (p = 0.077). All other outcomes were normal.

Outcome	Unit	E4.5(-) / R2.5		Natural pregnancy		P-value of means
		Mean per mouse (±SD)	Total	Mean per mouse (±SD)	Total	
Implants viable + non-viable fetuses	n	9.8 (±0.5)	78	9.0 (±0.9)	81	0.713
Viable fetuses	n	8.4 (±1.4)	67	8.7 (±1.2)	78	0.009[a]
Non-viable fetuses	n	1.4 (±1.5)	11	0.3 (±0.7)	3	0.027[a]
Losses	n	0.2 (±0.5)	2	0.0	0	0.713
Fetal weight	g	1.0 (±0.1)	-	1.0 (±0.07)	-	0.361
Placental weight	g	0.10 (±0.02)	-	0.12 (±0.01)	-	0.631
Fetal:placental weight ratio	-	9.1 (±1.5)	-	8.5 (±1.0)	-	0.077

a = significantly different p ≤0.05

Of the embryos that successfully implanted, a mean of 8.4 (SD±1.4) were viable fetuses and 1.4 (SD±1.5) non-viable fetuses (Table 28). Although the mean numbers of viable fetuses was only slightly reduced compared to natural mouse pregnancy (8.7, SD±1.2), this difference was significant (p = 0.009). Again, the small increase in the mean numbers of non-viable fetuses compared natural mouse pregnancy was significant (0.3 SD±0.7; p = 0.027). The remainder of transferred embryos resulted in a mean of 0.2 losses (SD±0.5), which were comparable to natural pregnancy (0.0; p = 0.713). The mean fetal (1.0 g, SD±0.1) and placental (0.1 g, SD±0.02) weights were comparable to natural mouse pregnancy (1.0 g, SD±0.07; 0.1 g, SD±0.01; p ≥ 0.361). The resulting mean fetal:placental ratio (9.1 SD±1.5) was increased compared to natural mouse pregnancy (8.5, SD±1.0; p = 0.077). Gross morphology and development was also comparable to natural mouse pregnancy (Figure 43).

Table 29 Grade allocation of E4.5(-) embryos asynchronously transferred into R2.5 recipient mice. Each recipient mouse represented one replicate, and was grouped by experimental series (Figure 15). Donor embryos were retrieved from a pool of prepubescent donor mice for each experimental series. Embryo grades, indicating stage of development and quality, were allocated as per Section 3.2.8 (Table 17). Mean numbers of implants were assessed on day-17 pc. Recipients 1, and 4 represent recipients 7 and 8 in Figure 42). Key: Recip, recipient; Exp, experimental; ET, embryo transfer; 2A – 1A, blastocyst; HgB, hatching; HdB, hatched.

Recip	Exp series	Numbers per recipient mouse				
		Embryo grade at time of ET (%)				Implants
		2A	1A	HgB	HdB	
1	1	1 (10)	-	9 (90)	-	9
2	1	-	-	10 (100)[a]	-	10
3	1	1 (10)	-	9 (90)	-	10
4	1	-	1 (10)	8 (80)	1 (10)	9
5	2	-	1 (10)	8 (80)	1 (10)	10
6	2	-	-	8 (80)	1 (10)	10
7	2	-	-	8 (80)	1 (10)	10
8	2	-	-	8 (80)	1 (10)	10

a = one B grade
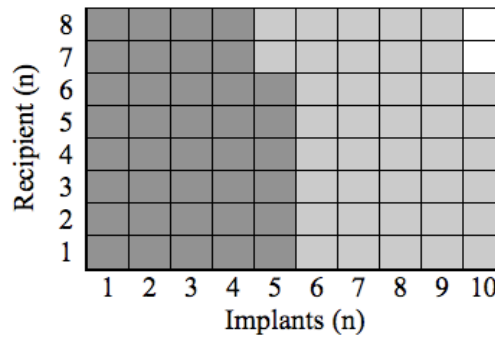
Figure 42 Numbers and location of implants following asynchronous transfer of E4.5(-) embryos (five per uterine horn) into eight R2.5 recipient mice. Features as per Figure 20. Recipient mice 1 – 6 resulted in maximum numbers of implants.
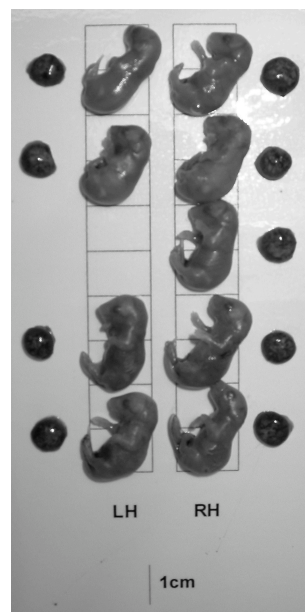
Figure 43 An example of gross morphology and development day-17 pc following asynchronous transfer of E4.5(-) embryos (five per uterine horn) into eight R2.5 recipient mice. Layout as per Figure 21. All fetuses normal weight for gestational age. Viable fetuses were observed in all recipient mice, resulting in a mean number of 8.4 per recipient mouse (SD±1.4).

4.4.2.3 Synchronous transfer of E3.5(-) embryos into R3.5 recipient mice

The experimental design leading up to the synchronous transfer of E3.5(-) embryos into R3.5 recipient mice, is illustrated in Figure 44. Ten replicates (recipient mice) were completed over three experimental series (three weeks; Figure 15). It is important to note that data presented is a second set of experimental series. The substantially low mean implantation rates exhibited in the first set of experimental series (0.8 implants per recipient mouse, SD± 0.03; 10 replicates; data not presented) prompted the need to repeat work, which is now presented in this section.

A mean implantation rate of 2.2 implants per recipient mouse (SD±2.3) resulted following the synchronous transfer of E3.5(-) embryos into R3.5 recipient mice. As seen in Table 30, the resulting implantation rate was significantly lower than natural mouse pregnancy (9.0, SD±0.9; $p < 0.001$). Sixty percent of recipient mice resulted in 2 – 6 implants per recipient mouse (Figure 45). The remaining 40% resulted in zero implants. A small amount of premature hatching was seen in transferred embryos (Table 31).

A mean of 1.3 (SD±1.8) resulted in viable fetuses and 0.9 (SD±1.1) resulted in non-viable fetuses (Table 30). While the mean numbers of viable fetuses was significantly reduced to natural mouse pregnancy (8.7, SD±1.2; $p < 0.001$), the mean numbers of non-viable fetuses was comparable (0.3, SD±0.7; $p = 0.139$). A mean of 7.8 embryos resulted in a loss (SD±2.3). Again, this mean value was significantly increased compared to natural mouse pregnancy (0.0; $p < 0.001$).

The mean fetal weight (0.8 g, SD±0.1) was significantly reduced compared to natural mouse pregnancy (1.0 g, SD±0.07; $p < 0.001$; Figure 46). In contrast, the mean placental weight (0.14g, SD±0.01) was significantly increased compared to natural mouse pregnancy (0.12 g, SD±0.01; $p < 0.001$), as can be seen clearly in Figure 47. The resulting mean fetal:placental ratio (5.5, SD±0.9) was significantly reduced compared to natural mouse pregnancy (8.5, SD±1.0; $p < 0.001$). Gross morphology and development were comparable to natural mouse pregnancy.
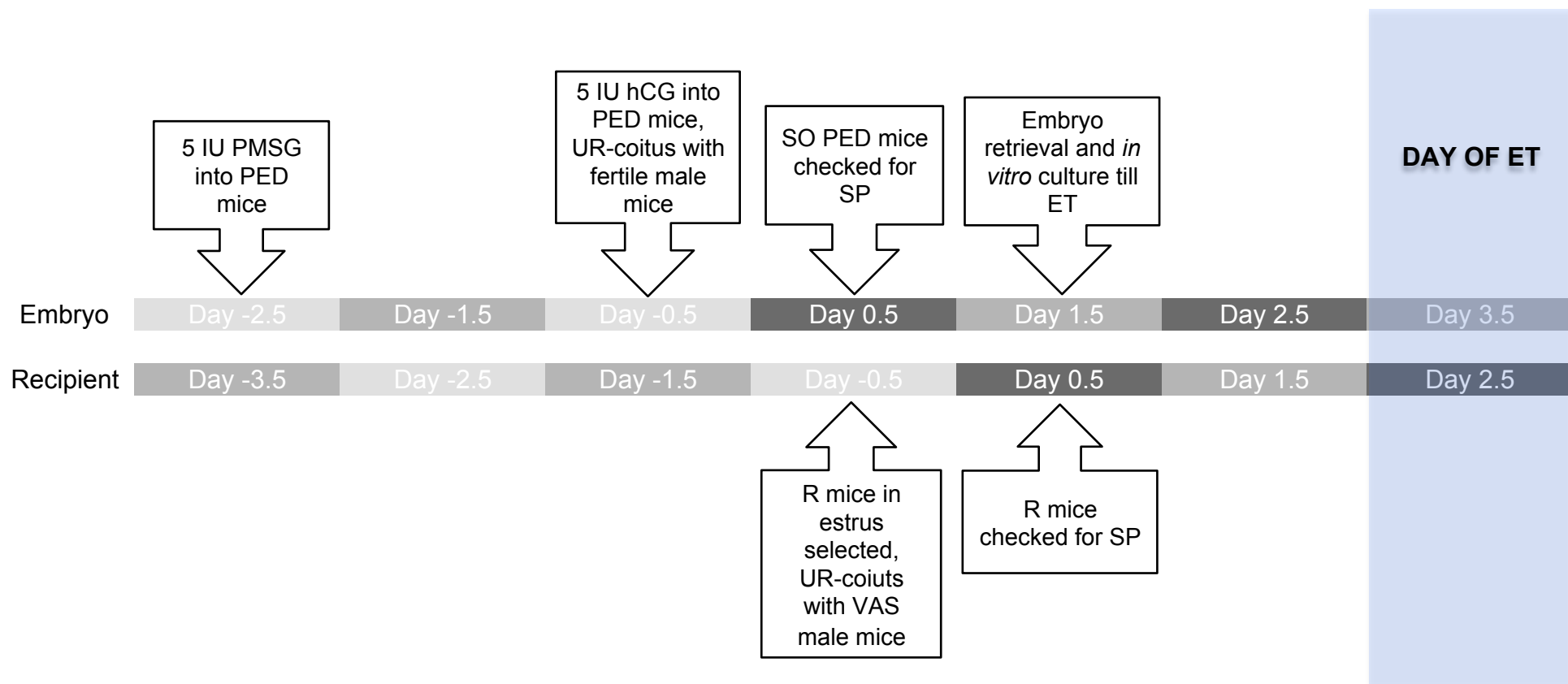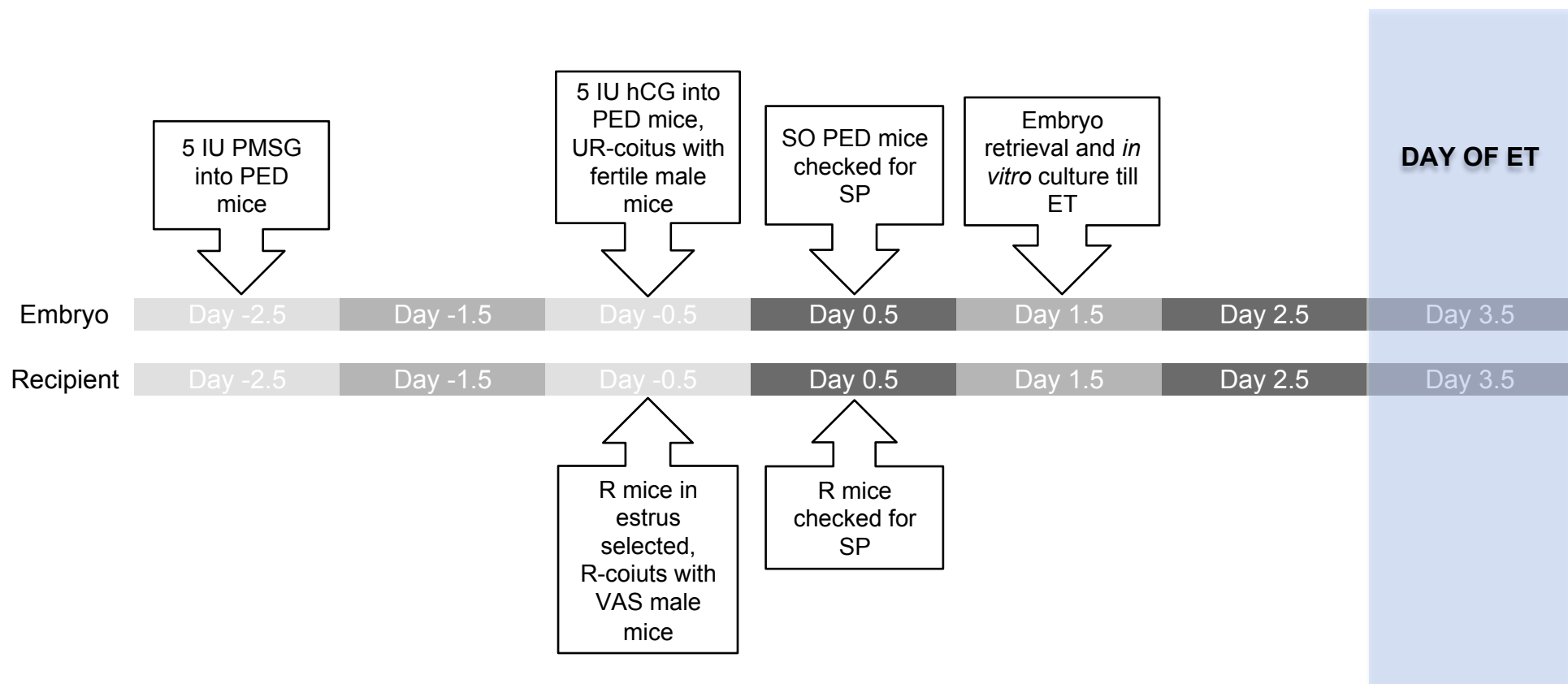
Figure 44 Schematic summarising the experimental design for the asynchronous transfer of E3.5(-) embryos into R3.5 recipient mice. Days are plus (+) or minus (–) of coitus. All other features as per Figure 24. Key: R, restricted.

Table 30 Mean outcomes of synchronous transfer of E3.5(-) embryos (five per uterine horn) into ten R3.5 recipient mice. Mean numbers of implants, viable fetuses, non-viable fetuses, and losses; and the mean fetal and placental weights day-17 pc. Mean numbers of implants, viable fetuses, mean fetal weight and fetal:placental ratios were significantly reduced compared to natural mouse pregnancy (p < 0.001). Mean numbers of losses and the mean placental weight were significantly increased (p < 0.001).

Outcome	Unit	E3.5(-) / R3.5		Natural pregnancy		P-value of means
		Mean per mouse (±SD)	Total	Mean per mouse (±SD)	Total	
Implants viable + non-viable fetuses	n	2.2 (±2.3)	20	9.0 (±0.9)	81	< 0.001[a]
Viable fetuses	n	1.3 (±1.8)	11	8.7 (±1.2)	78	< 0.001[a]
Non-viable fetuses	n	0.9 (±1.1)	9	0.3 (±0.7)	3	0.139
Losses	n	7.8 (±2.3)	70	0.0	0	< 0.001[a]
Fetal weight	g	0.8 (±0.1)	-	1.0 (±0.07)	-	< 0.001[a]
Placental weight	g	0.14 (±0.01)	-	0.12 (±0.01)	-	< 0.001[a]
Fetal:placental weight ratio	-	5.5 (±0.9)	-	8.51 (±1.0)	-	< 0.001[a]

a = significantly different p ≤0.05

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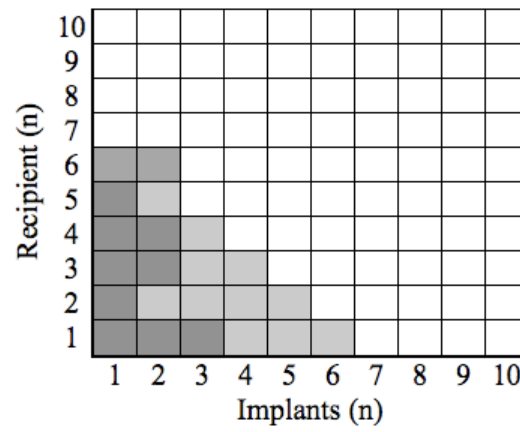
Figure 45 Numbers and location of implants on day-17 pc following synchronous transfer of E3.5(-) embryos (five per uterine horn) into ten R3.5 recipient mice. Features as per Figure 20. Recipient mice 7 – 10 resulted in no implants.

Table 31 Grade allocation of E3.5(-) embryos synchronously transferred into R3.5 recipient mice. Each recipient mouse represented one replicate, and was grouped by experimental series (Figure 15). Donor embryos were retrieved from a pool of prepubescent donor mice for each experimental series. Embryo grades, indicating stage of development and quality, were allocated as per Section 3.2.8 (Table 17). Mean numbers of implants were assessed on day-17 pc. Recipients 1, 2, 3, 4, 9 and 10 represent recipients 2, 1, 6, 3, 4 and 5 in Figure 45. Key: Recip, recipient; Exp, experimental; ET, embryo transfer; M, morula; 5A - 2A, A grade blastocysts.

Recip	Exp series	Numbers per recipient mouse (%)					Implants
		Embryo grade at time of ET					
		M[a]	5A	4A	3A	2A	
1	1	3 (30)	-	4 (40)	-	3 (30)[b]	5
2	1	8 (80)	-	1 (10)	1 (10)	-	6
3	1	3 (30)	-	4 (40)	3 (30)[b]	-	2
4	2	6 (60)	-	3 (30)[c]	-	-	4
5	2	7 (70)	-	2 (20)	1 (10)	-	0
6	2	8 (80)	-	1 (10)	1 (10)	-	0
7	3	7 (70)	-	4 (40)[d]	-	-	0
8	3	7 (70)	-	2 (20)[d]	1 (10)	-	0
9	3	6 (60)	-	1 (10)	2 (20)[d]	1 (10)[b]	3
10	3	5 (50)	-	3 (30)	-	2 (20)[d]	2

a = all morula A grade embryos
b = prematurely hatching, but cell integrity in tack
c = one B grade
d = one prematurely hatching, but cell integrity in tack
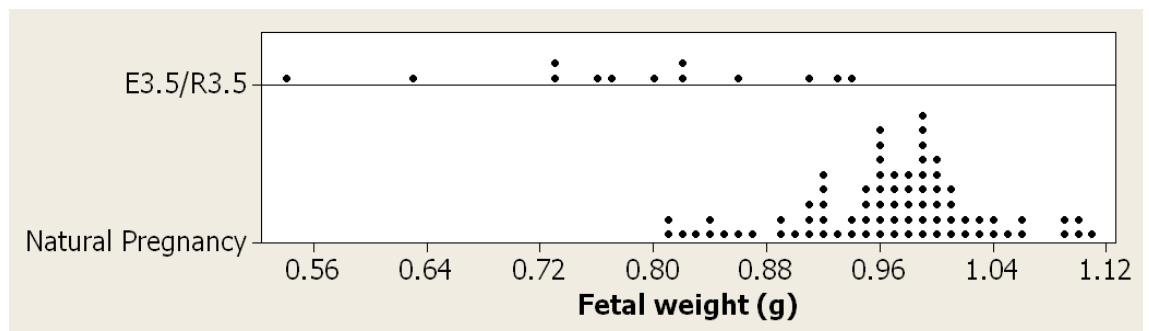
Figure 46 Fetal weights day-17 pc as a result of synchronous transfer of E3.5(-) embryos (five per uterine horn) into ten R3.5 recipient mice. Fetal weights were significantly reduced compared to natural pregnancy (p < 0.001).
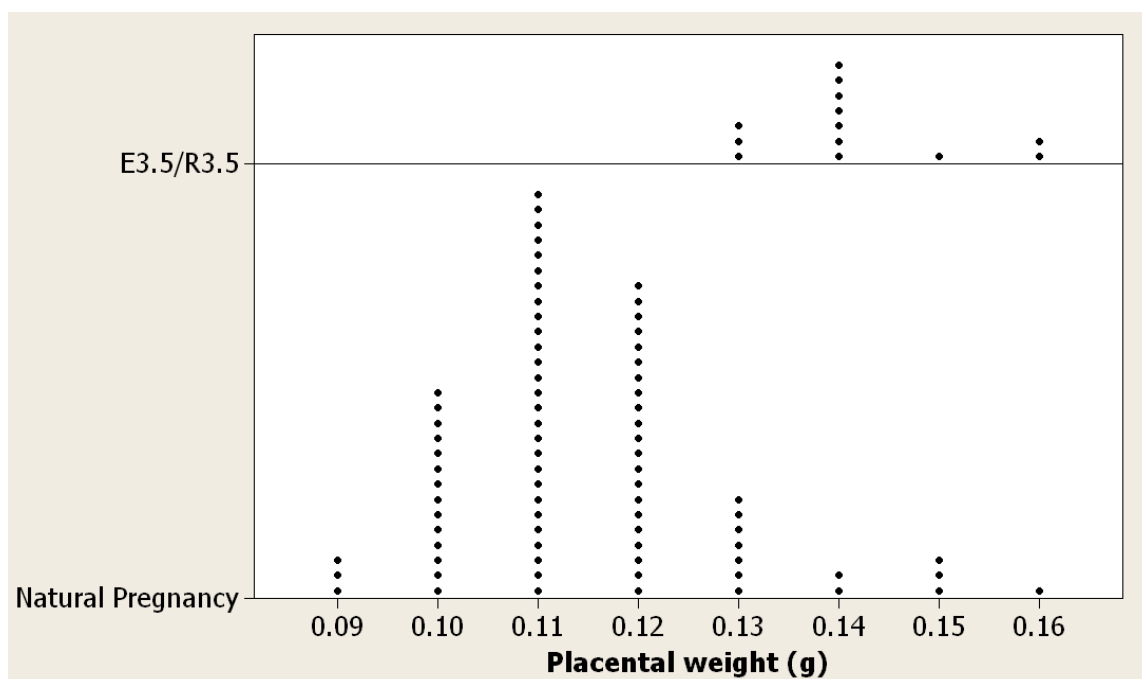
Figure 47 Placental weights day-17 pc as a result of synchronous transfer of E3.5(-) embryos (five per uterine horn) into ten R3.5 recipient mice. Placental weights were significantly increased compared to natural pregnancy (p < 0.001).

4.4.2.4 Asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice

Figure 48 summarises the experimental design leading up to the asynchronous transfer of E3.5(-) embryos into R4.5 recipient mice. Nine replicates were completed over two experimental series (two weeks; Figure 15).

Table 32 demonstrates that the asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice resulted in a mean implantation rate of 6.2 implants per recipient mouse (SD±2.6), which was significantly reduced compared to natural mouse pregnancy (9.0, SD±0.9; p < 0.001). All of recipient mice assessed resulted in implants, ranging from 2 – 10 implants per recipient mouse (Figure 49). Of those successfully implanted embryos, a mean of 4.3 viable fetuses resulted (SD±2.4), followed by a mean of 1.9 non-viable fetuses (SD±0.8; Table 32). While the mean numbers of viable fetuses was significantly reduced compared to natural mouse pregnancy (8.7, SD±1.2; p < 0.001), the mean numbers of non-viable fetuses was significantly increased (0.3, SD±0.7; p = 0.002). The remainder of transferred embryos resulted in a loss (3.8, SD±2.6), which was significantly increased compared to natural pregnancy (p < 0.001). All transferred embryo were A-grade quality, with 92% (83/90) classified as hatching or hatched blastocysts (Table 27).

The mean fetal weight (0.9 g, SD±0.1) was comparable to natural mouse pregnancy (1.0 g, SD±0.07; p = 0.093). In contrast, the mean placental weight (0.14 g, SD±0.03) was significantly increased compared to natural mouse pregnancy (0.12 g, SD±0.01; p < 0.001; Figure 50). The resulting mean fetal:placental ratio (6.8, SD±1.4) was significantly lower than natural mouse pregnancy (8.5, SD±1.0; p < 0.001). Gross morphology and development were comparable to natural mouse pregnancy.
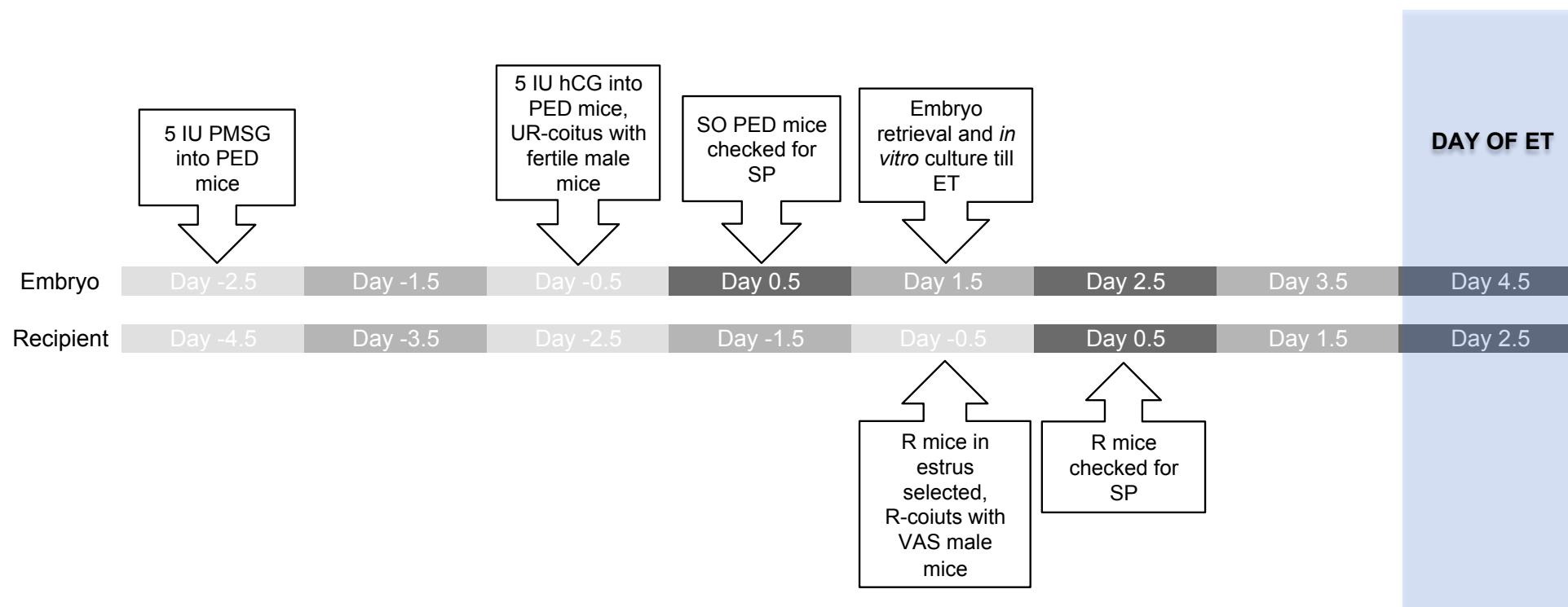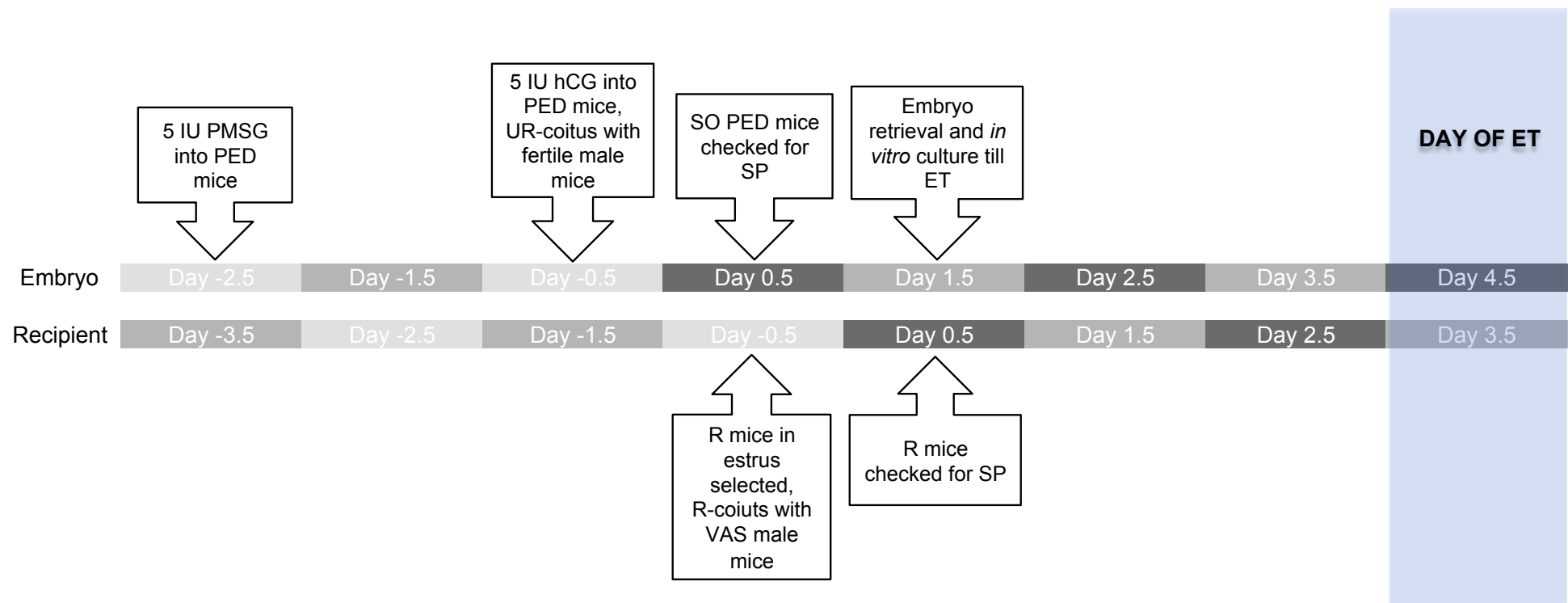
Figure 48 Schematic summarising the experimental design for the asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice. Days are plus (+) or minus (–) of coitus. All other features as per Figure 24. Key: R, restricted.

Table 32 Mean outcomes of asynchronous transfer of E4.5(-) embryos (five per uterine horn) into nine R3.5 recipient mice. Mean numbers of implants, viable fetuses, non-viable fetuses; and mean fetal and placental weights day-17 pc. Mean numbers of implants and viable fetuses and fetal:placental weight ratios were significantly decreased compared to natural mouse pregnancy (p < 0.001). Mean numbers of non-viable fetuses, losses and the mean placental weight were significantly increased (p < 0.001). The small reduction in mean fetal weight demonstrated a strong tendency towards significance (p = 0.093).

Outcome	Unit	E4.5(-) / R3.5		Natural pregnancy		P-value of means
		Mean per mouse (±SD)	Total	Mean per mouse (±SD)	Total	
Implants viable + non-viable fetuses	n	6.2 (±2.6)	56	9.0 (±0.9)	81	< 0.001[a]
Viable fetuses	n	4.3 (±2.4)	39	8.7 (±1.2)	78	< 0.001[a]
Non-viable fetuses	n	1.9 (±0.8)	17	0.3 (±0.7)	3	0.002[a]
Losses	n	3.8 (±2.6)	34	0.0	0	< 0.001[a]
Fetal weight	g	0.9 (±0.09)	-	1.0 (±0.07)	-	0.093
Placental weight	g	0.14 (±0.03)	-	0.12 (±0.01)	-	< 0.001[a]
Fetal:placental weight ratio	-	6.8 (±1.4)	-	8.51 (±1.0)	-	< 0.001[a]

a = significantly different p ≤0.05

Table 33 Grade allocation of E4.5(-) embryos asynchronously transferred into R3.5 recipient mice. Each recipient mouse represented one replicate, and was grouped by experimental series (Figure 15). Donor embryos were retrieved from a pool of prepubescent donor mice for each experimental series. Embryo grades, indicating stage of development and quality, were allocated as per Section 3.2.8 (Table 17). Mean numbers of implants were assessed on day-17 pc. Recipients 1 through to 10 represent recipients 2, 9, 6, 8, 1, 3, 5, 4, and 7 in Figure 49. Key: Recip, recipient; Exp, experimental; ET, embryo transfer; 2A – 1A, blastocyst; HgB, hatching; HdB, hatched.

| Recip	Exp series	Numbers per recipient mouse (%)				Implants
		Embryo grade at time of ET				
		2A	1A	HgB	HdB	
1	1	2 (20)	2 (20)	6 (60)	-	8
2	1	-	-	10 (100)	-	2
3	1	-	-	10 (100)	-	5
4	2	-	-	10 (100)	-	4
5	2	-	1 (10)	8 (80)	1 (10)	10
6	2	-	-	10 (100)	-	8
7	3	-	-	10 (100)	-	7
8	3	1 (10)	1 (10)	8 (80)	-	8
9	3	-	-	10 (100)	-	4
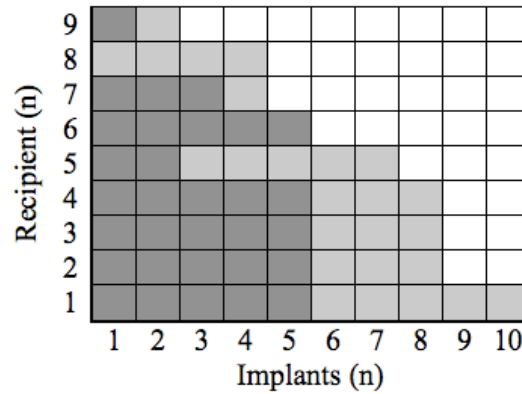
Figure 49 Numbers and location of implants day-17 pc following asynchronous transfer of E4.5(-) embryos (five per uterine horn) into nine R3.5 recipient mice. Features as per Figure 20. Recipient mice resulted in 2 – 10 implants per recipient mouse. No significant difference between left and right uterine horns (p = 0.893).
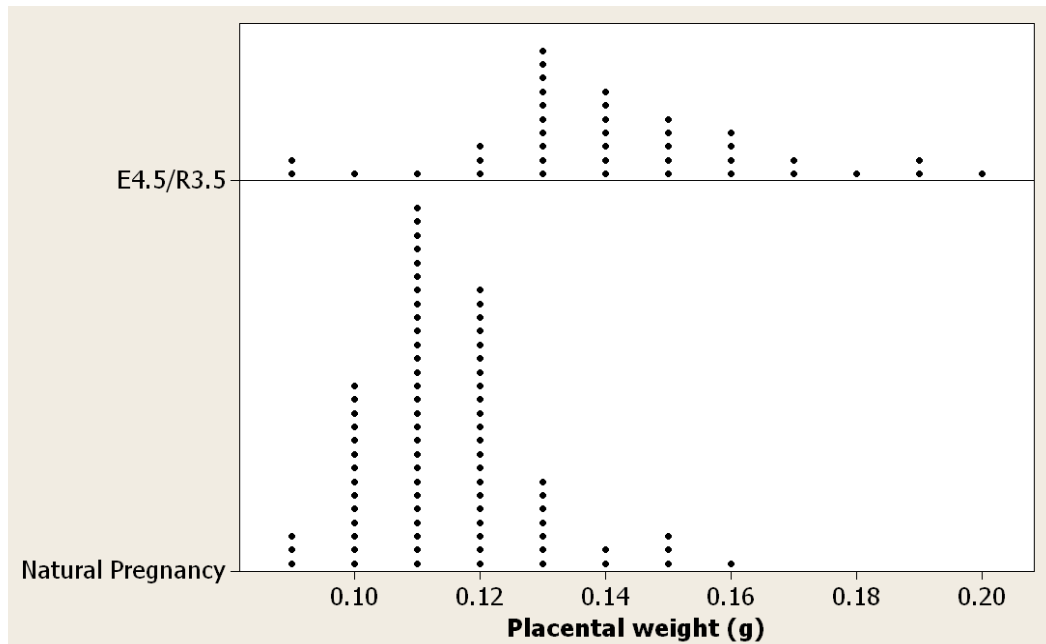
Figure 50 Placental weights day-17 pc following the asynchronous transfer of E4.5(-) embryos (five per uterine horn) into nine R3.5 recipient mice. A significant increase in the mean placental weight was observed compared to natural mouse pregnancy (p < 0.001).

4.4.3   Interpretation for synchronous and asynchronous ET experiments:

The four different synchronous and asynchronous ET scenarios assessed within this research could be categorised into two groups.   The first group included the asynchronous transfer of E3.5(-) or E4.5(-) embryos into R2.5 recipient mice, which resulted in optimal implantation rates. In contrast, the second group, which included the synchronous and asynchronous transfer of E3.5(-) or E4.5(-) embryos into R3.5 recipient mice, exhibited suboptimal implantation rates. Subsequently, each group was assessed against the corresponding criteria in order to select the most suitable optimal and suboptimal models.

As demonstrated in Figure 51 and Figure 52, the asynchronous transfer of E3.5(-) or E4.5(-) embryos into R2.5 recipient mice resulted in mean implantation rates of 9.4 and 9.8 implants per recipient mouse.   This level of implantation was considered optimal, and was comparable to or greater than natural mouse pregnancy (9.0 implants per mouse, Table 20). Not only did this indicate that implantation was normal, but also any negative consequences of manipulated embryos would be easily observed within either scenario.   Consequently, the asynchronous transfer of E3.5(-) or E4.5(-) embryos into R2.5 recipient mice met the first criterion used to help select the most suitable Optimal Model.
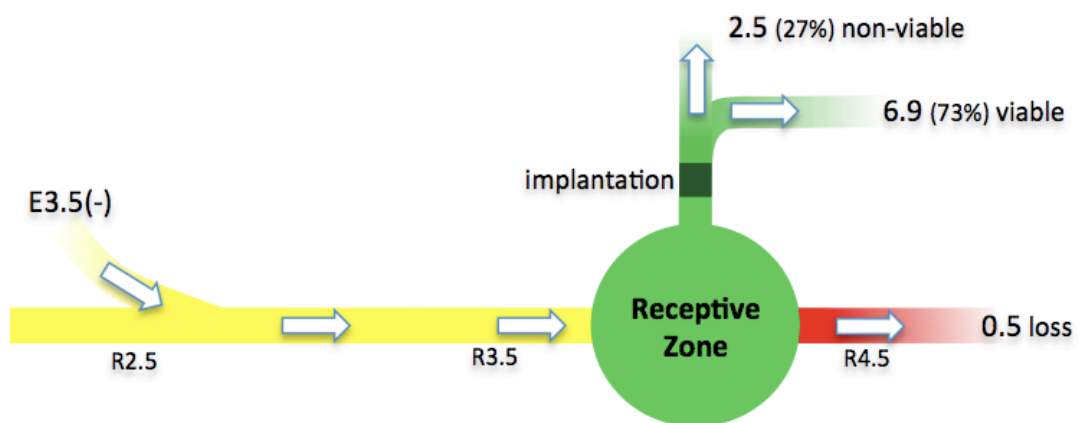
Figure 51 Schematic representation of the Optimal Model mean outcomes. For every 10 *in vitro* cultured E3.5(-) embryos transferred into R2.5 recipient mice, a mean of 9.4 embryos successfully implanted. A mean of 6.9 (73%) led to viable fetuses and 2.5 (27%) led to non-viable fetuses. The remaining embryos failed to implant prior to end of the receptive zone, leading to a mean of 0.5 losses.
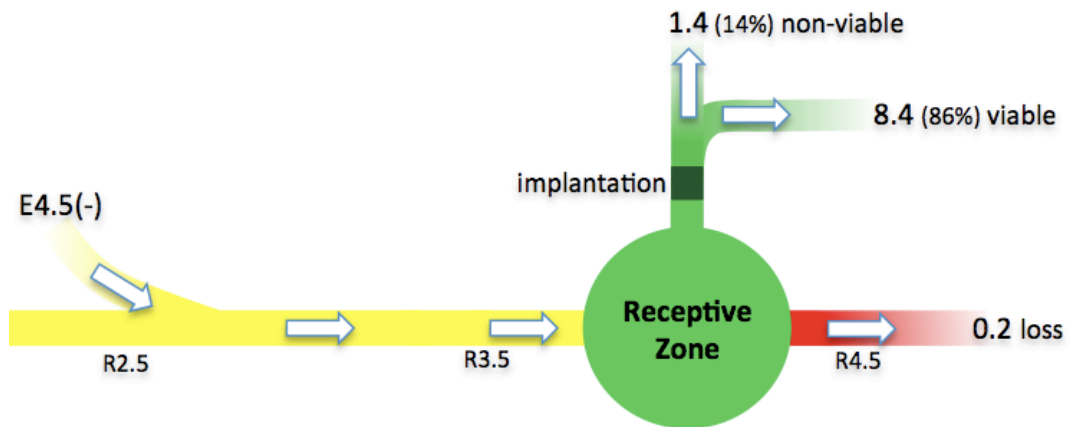
Figure 52 Schematic representation of asynchronous transfer of E4.5(-) embryos into R2.5 recipient mice mean outcomes. A mean of 9.8 embryos successfully implanted. Means of 8.4 (86%) fetuses were viable and 1.4 (14%) were non-viable fetuses. The remainder of embryos failed to implant prior to end of the receptive zone, leading to a mean of 0.2 losses.

Both the asynchronous transfer of E3.5(-) and E4.5(-) embryos into R2.5 recipient mice exhibited viability within 73% and 86% of fetuses. Both percentage values exceeded the minimum requirement of 70% fetal viability, which was specified by the second criterion used to help select the most suitable Optimal Model. The asynchronous transfer of E4.5(-) embryos into R2.5 recipient mice attained 86% fetal viability, which was significantly reduced compared to natural mouse pregnancy (97%, $p = 0.009$). In contrast, E3.5(-) embryos resulted in 73% viability, which was significantly different to natural mouse pregnancy (97%, $p < 0.001$). Despite the reduction in fetal viability in either case, values exceeded 70%. Within this research, a minimum of 70% viability simply ensured sufficient numbers of fetuses for examination. This in turn kept the experimental numbers of recipient mice to a minimum and would not impact on overall implantation rates, which were the focus of this research. Therefore, based on the selection criteria, both ET scenarios were considered as suitable Optimal models.

It was necessary however, to only evaluate one optimal model for its ability to detect negative consequences of manipulated embryos. Given the amount of available literature surrounding the asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice, thus allowing greater comparison, this ET scenario was chosen as the most suitable of the two models (Doyle, et al., 1963; Lane & Gardner, 1994; McLaren & Michie, 1955; Wakuda, et al,. 1999). For the remainder of research, this model would be referred to as the Optimal Model. Section 4.5 would thus address the Optimal Model's ability to assess negative consequences of manipulated embryos.

The synchronous and asynchronous transfer of E3.5(-) or E4.5(-) embryos into R3.5 recipient mice belonged to the second group of ET scenarios, which demonstrated suboptimal implantation rates. For this reason, the selection criteria associated with suboptimal implantation rates would form the basis for assessment. Such criteria included: 1) the mechanism of action on implantation would need to prevent implantation (pre-implantation loss) rather than abortive (post-implantation loss); and 2) demonstration of an approximate mean implantation rate of 6 implants per recipient mouse for every 10 embryos transferred (± 1 implant), which would demonstrate both implantation capability and enable positive consequences of manipulated embryos to be detected.

The synchronous transfer of E3.5(-) embryos into R3.5 recipient mice exhibited prevented implantation (Figure 53). Not only did the model result in a suboptimal implantation rate of 2.2 implants per recipient mouse, but also increased mean numbers of losses (7.8) compared to natural mouse pregnancy (0.0). Furthermore, mean numbers of non-viable fetuses (0.9) remained relatively unchanged. As a result, the synchronous transfer of E3.5(-) embryos into R3.5 recipient mice met the first criterion used as a basis for selection of the most suitable suboptimal model.

Although the mean implantation rate was suboptimal, 2.2 implants per recipient mouse (SD±2.3) was substantially lower than 6 implants per recipient mouse (± 1 implant). Moreover, while 60% of recipient mice resulted in 2 – 6 implants per recipient mouse, 40% of recipient mice did not demonstrate an implantation capability. Consequently, it was difficult to determine whether the lack of implants was due to implantation incapability of that mouse or whether the model design had prevented implantation. At first glace the mean implantation rate appeared low considering the scenario's synchrony is most comparable to natural mouse pregnancy. Technical issues surrounding embryo culture and resulting quality was questioned. While each replicate (recipient mouse) had it's own set of microdrops and utilised embryos that were retrieved from pool of donor mice, some embryos presented premature hatching. However, these embryos represented only 10% of the total numbers of embryos transferred (100). In addition, no pattern appeared to be associated with premature hatching and low or zero implantation (Table 31).

Presented data was also the second set of experimental series. The first set of ten replicates (three experimental series) presented even lower mean implantation rates

(0.8 implants per recipient mouse, SD± 0.03; data not presented). When presented data was taken in context of the other asynchronous ET scenarios, the resulting pattern was comparable to McLaren and Michie (1955; Figure 11 and Figure 12). This pattern will be discussed in greater detail within Section 6.1.1. If the synchronous transfer of E3.5(-) embryos into R3.5 recipient mice were utilised, the numbers of mice required to show any significant improvement would be substantially higher than the other models available. Nevertheless, the synchronous ET scenario did not achieve 6 implants per recipient mouse (± 1 implant), and therefore, did not meet the second criterion.

In comparison to natural pregnancy, a significantly reduced mean fetal weight (0.8 g) and increased placental weight (0.14 g) suggested poor intrauterine growth. This was supported by a substantially lower mean fetal:placental ratio (5.5, SD±0.9) compared to natural mouse pregnancy (8.5, SD±1.0; $p < 0.001$), which suggested poor placental efficiency. Yet, no clear correlation between small fetal weight and large placental weight was observed. Nevertheless, this data reflected post-implantation events, which were not considered important in suboptimal model selection. Therefore, data indicated that the synchronous transfer of E3.5(-) embryos into R3.5 recipient mice should not be assessed for its potential to detect positive consequences of manipulated embryos.
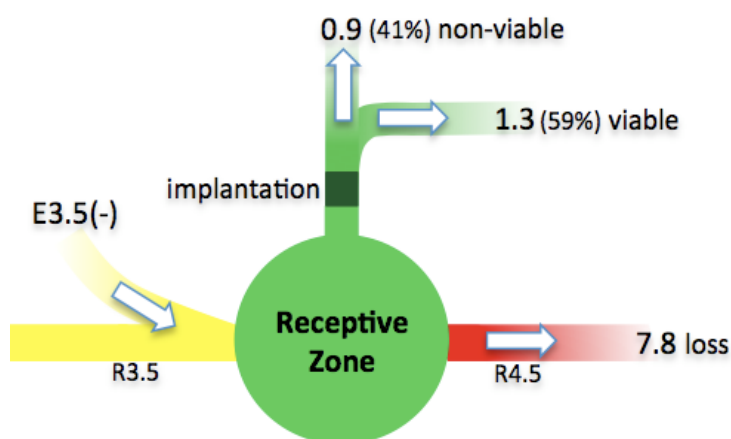
Figure 53 Schematic representation of synchronous transfer of E3.5(-) embryos into R3.5 recipient mice mean outcomes. For every 10 E3.5(-) embryos transferred into R3.5 recipient mice, 2.2 successfully implanted. A mean of 1.3 led to viable fetuses and 0.9 led to non-viable fetuses. The remainder of embryos failed to implant, leading to a mean of 7.8 losses.

The asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice also demonstrated prevented implantation. Following ET, as suboptimal implantation rate

of 6.2 implants per recipient mouse resulted, which reflected the increased mean numbers of losses (3.8; Figure 54). Interestingly, the mean numbers of non-viable fetuses (1.9) were increased in comparison to natural pregnancy (0.3). Nevertheless, 69% of fetuses were viable, which was comparable to the Optimal Model (73%). For this reason, the small increase in non-viable fetuses was not a concern. Therefore, the asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice met the first criterion used to define a suboptimal model.
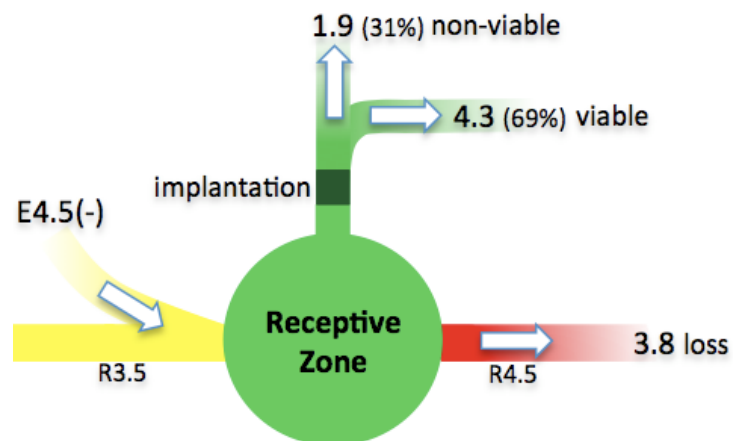
Figure 54 Schematic representation of asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice mean outcomes. Out of every 10 E4.5(-) embryos transferred into R3.5 recipient mice, 6.2 resulted in a successful implant. A mean of 4.3 implants (69%) led to viable fetuses and 1.9 (31%) led to non-viable fetuses. A mean of 3.8 losses accounted for the remainder of embryos.

The second criterion was only met in part. While a mean implantation rate of 6.2 implants per recipient mouse was more comparable to 6 implants per recipient mouse, mouse-to-mouse variability (± 4 implants) exceeded the allowed variability (± 1 implant). Implantation was observed in all recipient mice, where numbers of implants ranged from 2 through to 10 (potential maximum). Eighty percent of those recipient mice resulted in 4 - 8 implants per recipient mouse. Yet, 30% of recipient mice exhibited optimal implantation rates. Such mouse-to-mouse variability raised concerns regarding technical issues surrounding embryo culture and resulting quality. As in other ET scenarios, each recipient mouse was it's own replicate, grouped in experimental series. Each replicate had it's own set of culture microdrops and embryos were selected from a pool of donor mice. Although a small amount of variation was seen in stage of embryonic development (2A through to HdB), those recipients that received less mature embryos (2A – 1A) achieved 8 implants per recipient mouse or higher (Table 33). For these reasons, it was unlikely embryo culture

or stage of development was responsible for the inconsistent implantation rates observed.

Interestingly, while the mean fetal weight (0.9 g) was comparable to natural mouse pregnancy (1.0 g), the mean placental weight (0.14 g) was significantly increased (0.12 g; $p < 0.001$). This resulted in a reduced mean fetal:placental ratio (6.8, SD±1.4) compared to natural mouse pregnancy (8.5, SD±1.0; $p < 0.001$). Normally low fetal:placental weight ratios suggest poor intrauterine growth and low placental efficiency. However, mean fetal weight was considered normal. It is possible that due to the reduced numbers of implants, fetuses had more room to grow and could compensate placental inefficiency. While, Coan et al. (2008) did demonstrated an influence of placental weight and litter size day-15.5 pc of natural mouse pregnancy, this influence had disappeared by day-18.5 pc. In hindsight, further examination of placental surface area, thickness, capillary length and diameter, may have indicated placental efficiency more effectively (Coan, Fergugson-Smith, & Burton, 2004). In addition, if a duplicate group of recipient mice were allowed to give birth, postnatal growth trajectories would have indicated any long-term impact of the low fetal:placental weight ratio. Nonetheless, fetal and placental weights did not form the basis for the Suboptimal model's selection.

Although the asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice did not meet acceptable levels of mouse-to-mouse variability (± 1 implants), it held the most promise of all the models that exhibited low levels of implantation. The model was therefore referred to as the Suboptimal Model for the remainder of research. Subsequently, experiments assessing the model's ability to detect positive consequences of manipulated embryos were initiated (Section 4.6). However, the model's consistency continued to be troubling. Combined with the mouse-to-mouse variability observed within all other models that achieved suboptimal implantation rates, the need to gain a more in-depth understand of implantation consistency became apparent. For that reason, the research in Chapter 5 addresses the beginning of endometrial receptivity within natural mouse pregnancy, and synchronous and asynchronous ET, which highlighted the variables behind implantation consistency.

4.5 Ability of Optimal Model to detect negative consequences of manipulated embryos

Embryo manipulation, via an assortment of different molecules, offers an opportunity to improve IVF implantation rates. However, prior to their commercial use within humans, many preliminary studies, which include proof of principle, are required. Murine *in vivo* implantation models have provided a useful platform for such foundational studies. Not only do these models provide an insight into a live-animal scenario, but they can also enable increased understanding of embryo manipulation safety and help determine the potential embryo manipulation holds in improving suboptimal implantation rates.

As still in Chapter 4, standardization of *in vivo* implantation murine models that are suitable to explore the potential of embryo manipulation require both consistent mean implantation rates and an ability to detect consequences of that intervention. Models that achieve optimal implantation rates could be used to assess embryo manipulation for any detrimental effect *in vivo*. A reduction in mean implantation rates, fetal viability and mean fetal and placental weights could suggest that the specific manipulation had a negative effect on the embryo. In the context, of this research, any manipulation resulting in normal ET outcomes was considered safe.

Within this research, the asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice (Section 4.4; Figure 39) was selected as the Optimal Model. As described above, it was important to determine whether the Optimal model could detect negative consequences of manipulated embryos before it was used to explore the potential embryo manipulation held as an IVF-intervention. Therefore, molecules used to explore the Optimal model's ability were not necessarily selected for their involvement in implantation, but for their potential to measure negative consequences. In other words, molecules were simply used as tools within this research. However, those molecules that were associated with implantation, and resulted in normal ET outcomes, were considered to explore the Suboptimal model's ability to detect positive consequences of embryo manipulation.

Molecules fell into two categories: 1) those that spontaneously inserted into the cellular membrane of embryos through KODE™ Technology; and 2) non-insertion molecules that were free within the incubation media, for example biotinylated ganglioside (BioG). KODE™ Technology facilitates cell surface modification and visualization via the insertion of synthetic constructs, which consist of a functional head group (F), a spacer (S), and a lipid tail (L) (Figure 43; Blake, Bovin, Bess & Henry, 2011). The

technology exploits the ability of naturally occurring glycolipids to spontaneously insert into the phospholipid bilayer of cellular membranes (Blake, et al., 2011). While naturally occurring glycolipids have limited solubility within aqueous solutions, the spacer found within FSL-constructs enables high solubility, yet firm insertion into cellular membranes (Blake, et al., 2011). A number of biological surfaces have been successful manipulated via FSL-constructs, some of which include those found within murine embryos, human spermatozoa, zebra fish, murine and human epithelial endometrial cells, murine and human red blood cells and virions (Blake, et al., 2011).
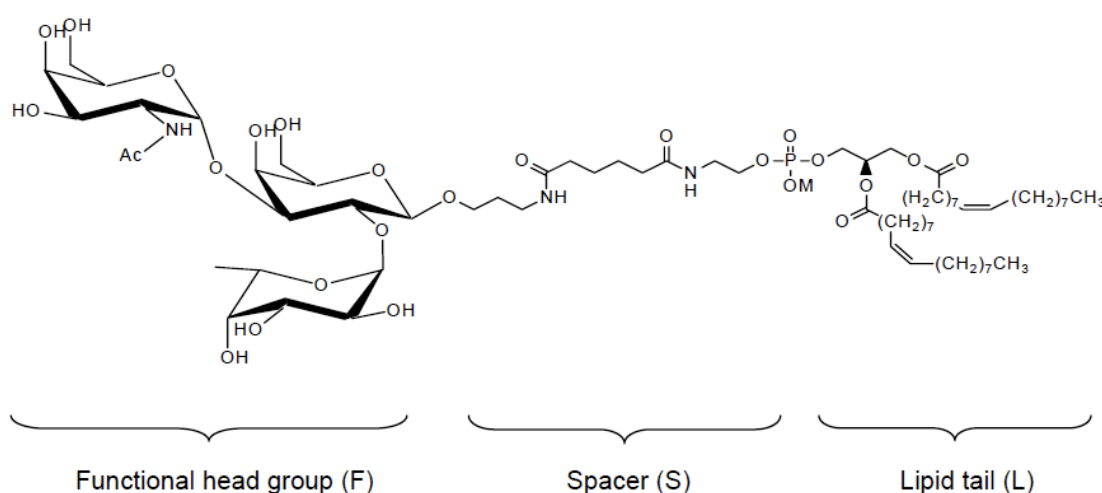
Figure 55 Schematic of a FSL-construct. Within this example, the functional head group (F) is the blood group A trisaccharide. Sourced from Williams (2008).

At the time of this research, FSL-A, FSL-Le^Y, FSL-HA$_{high}$, and FSL-HA$_{mid}$ were available and kindly donated by KODE Biotech Ltd to manipulate murine embryos. It should be noted that the concentrations used within this research exceed the recommendations from Blake et al. (2011). This information was not available at the time of research. For this reason, it is possible that negative consequences observed were be due to high concentration levels rather than the functional head group (F) of FSL-constructs. Nonetheless, the FSL-molecules could still be used as tools to investigate the ability of the Optimal and Suboptimal Models to detect negative or positive consequences (Sections 4.5 and 4.6). Specific concentrations will be discussed in each section. Embryo culture media was used as diluent for all molecules (including BioG).

Within Sections 4.1 – 4.4, recipient mice were considered as the experimental unit. Embryo transfer outcomes from individual recipient mice and overall means were

presented. In contrast, donor embryos within Sections 4.5 and 4.6 received the "treatment" and therefore have been assigned as the experimental unit. The measurable outcome was successful or failed implantation for each donor embryo. As a result, it was more appropriate to express collated data as a percentage of the total numbers of donor embryos transferred (implantation frequency), rather than means and individual data from each recipient mouse.

Prior to the transfer of manipulated embryos, it was first necessary to investigate whether two different groups of embryos could be assessed within the same recipient mouse (Section 4.5.1). If possible, experimental efficiency and robustness would be improved.

4.5.1 Incidence of transmigration

The transfer of two differently manipulated embryos into the same recipient mouse offered two benefits. Firstly, recipient mice could act as their own control, thus reducing experimental variability. Secondly, statistical power would be increased, resulting in reduced numbers of experimental mice. Nonetheless, at the time this research such practice was still not popular, nor recommended by researchers. Their reasons lay in the assumed risk of transmigration, the movement of embryos from one horn to the other, or their inability to identify embryos from different treatments.

Prior to the work of Rulicke et al. (2006), research exploring the nature of transmigration within mice was limited (McLaren & Michie, 1955; Runner 1951). Therefore, due to the close relationship between mice and rats, it was assumed that these species would share a similar transmigration pattern. In 2006, Rulicke et al. reported promising results, where only a single transmigrated embryo following the transfer of 256 2-cell embryos (16 per uterine horn) into the left or right uterine horn of 16 recipient mice. It appeared that the partial septum within mice uteri was sufficient to prevent transmigration. However, this information was not available at the time Section 4.5.1 was completed (2005). In addition, operator differences needed to be considered. Therefore, it was important to determine the incidence of transmigration within the context of this research. Data are used to confirm the final experimental design utilised to assess the ability of the Optimal Model.

4.5.1.1 Methodology Overview:

Power analysis indicated that eight or more recipient mice would achieve 80% statistical power. Embryo production and collection from pre-pubescent donor B6CBAF1/J mice was performed on day-1.5 pc (Section 2.2.7). Embryos were then *in vitro* cultured in 5% CO_2 at 37°C for two days. Pseudo-pregnant B6CBAF1/J recipient mice were produced by unrestricted-coitus with vasectomised CBA/J male mice of proven sterility (Section 3.2.3). Restricted-coitus was not utilized as the specialised cages required had not yet been introduced at the time of experimentation.

Immediately prior to ET, R2.5 recipient mice were anaesthetised using avertin anaesthetic. Five E3.5(-) embryos were transferred into the left-hand uterine horn of each recipient mouse. Grades of transferred embryos ranged from M to 3A embryos (Section 3.2.8). All were A-grade embryos. As a control, media alone (< 0.5 μL) was transferred into the right-hand uterine horn of each recipient mouse. A total of 11 replicates (11 recipient mice) were completed over five experimental series (5 weeks; Figure 16). Female mice were euthanased 8 – 10 days pc, where the uteri excised, and the number of implants in each uterine horn was recorded (Section 3.2.11). Numbers of implants in left and right horns were compared using Wilcoxon signed rank test.

4.5.1.2 Results:

Following the transfer of E3.5(-) embryos into the left-hand horn of R2.5 recipient mice no incidence of transmigration was observed (Table 34). Of the 55 embryos transferred, 54 (98%) successfully implanted. All of those implants were observed within the left-hand uterine horn of each recipient mouse. No physiological abnormalities were observed within the right-hand uterine horn after the transfer of media alone.

Table 34 Incidence of transmigration within the Optimal Model. Total numbers of implants and their location on days 8 – 10 pc within the Optimal Model. Data collated from 11 recipient mice. No transmigration was observed.

Uterine Horn	Implants / embryos transferred (n)
Left	54 / 55[a]
Right	0 / 0[a]

a = significantly different, p = 0.004

4.5.1.3 Interpretation

Implantation data indicated that the risk of transmigration was negligible. Therefore, the transfer of two different groups of embryos into the same recipient mouse was sound and statistically robust. Such experimental design was utilised in subsequent sections.

4.5.2 FSL-A manipulated embryos

The functional head-group of FSL-A was the A trisaccharide antigen of the ABO blood group system, found on the surface of human erythrocytes (Figure 55; Figure 56). The A-antigen is not known to be involved in implantation and has no native ligands on mouse endometrium (Aplin, 1997, 2000; Armant, 2005; Carter 2007b). Therefore, FSL-A represented a non-active FSL-structure within the mouse reproductive system.
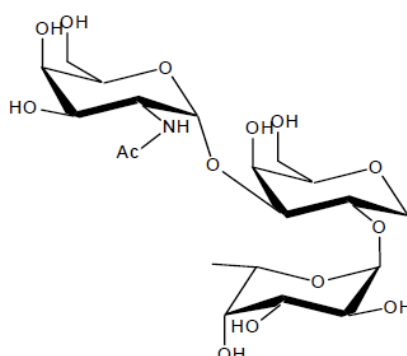
Figure 56 Schematic of blood group A trisaccharide antigen. Adapted from Figure 55.

While Carter demonstrated normal blastocyst expansion following their manipulation with FSL-A (Table 35), the impact on implantation rates post-ET was unknown (2007b).

Therefore, as per Cater (2007), E3.5(-) embryos were manipulated with 1 mg/mL FSL-A for two hours and transferred into R2.5 recipient mice (Optimal model).

Table 35 Development of zona–free embryos following treatment with FSL-A within Carter (2007b). Donor embryos were collected from superovulated prepubescent B6CBAF1/J mice on day-3.5 pc (E3.5). Zona pellucida were immediately removed using 5% pronase (in M2 media, Sigma-Aldrich, Cat# M7167, hepes buffered) and were subsequently incubated for 2 hours in 1 mg/mL FSL-A or media alone (M16, Sigma-Aldrich, Cat# M7292, bicarbonate buffered). Following treatment, embryos were incubated for a further twenty-four hours (50µL M16 microdrops, 5% CO_2). Numbers of expanded blastocysts[a] were then counted.

	Treatment		P-value
	FSL-A	Media alone	
Numbers of embryos treated	14	18	-
Numbers of expanded blastocysts[a] (%)	13 (93)	16 (89)	> 0.05[b]

a = includes 1A or 2A embryos as per Section 3.2.8.
b = no significant difference calculated by Carter (2007b).

4.5.2.1 Methodology overview:

Power analysis was performed using PASS, a NCSS statistical software programme. With the assistance of an AUT Statisics Lecturer, PASS calculated 80% power would be achieved with eight or more recipient mice. This remained standard for the remainder of Section 4.5 and 4.6. Donor embryos were retrieved from pre-pubescent donor B6CBAF1/J mice on day-1.5 pc (Section 3.2.7). Embryos were then *in vitro* cultured in 5% CO_2 at 37°C for two days. Unrestricted-coitus with vasectomised CBA/J male mice, of proven sterility, was used to generate pseudo-pregnant B6CBAF1/J recipient mice (Sections 3.2.2 and 3.2.3). Restricted-coitus was not utilized, as the specialised cages required had not yet been introduced at the time of experimentation.

On the same day as ET, E3.5(-) embryos were incubated with 1 mg/mL FSL-A or with culture media alone for two hours (Sections 3.1.2.3 and 3.2.9, Figure 17). Five FSL-A manipulated embryos were transferred into one uterine horn and five unmanipulated embryos (media alone) into the other uterine horn of each recipient mouse. The selection of embryos, the uterine horn and the order in which groups were transferred was randomised to remove bias. Based on Figure 17, a total of 13 replicates (recipient mice) were completed over six experimental series (six weeks).

Seventeen days pc, female mice were euthanased and assessed as per Section 3.2.11. The numbers of viable and non-viable fetuses, and losses were recorded. Photos were taken of intact uteri, followed by the excised fetuses and their corresponding placentae. Each fetus and placenta was weighed, and gross morphology and development were assessed visually. Binary logistic regression and general linear modelling were used to confirm any significant differences between FSL-A manipulated and unmanipulated embryos. Standard deviation was used to indicate mouse-to-mouse variation in fetal and placental weights.

4.5.2.2 Results:

Following the transfer of FSL-A manipulated embryos, 62 of the 65 transferred (95%) resulted in an implant (Table 36). This was identical to number of unmanipulated embryos (media alone) that resulted in an implant (62/65, 95%; $p = 0.717$). As seen in Figure 57, 48 implants resulting from FSL-A manipulated embryos were viable (74%), which were comparable to those resulting from unmanipulated embryos (44/65, 68%; $p = 0.492$). In contrast, the numbers of non-viable implants resulting from FSL-A manipulated (14/65, 22%) and unmanipulated embryos (18/65, 28%) were significantly different ($p = 0.041$). The remaining 4 FSL-A manipulated embryos (3%) resulted in a loss, which was identical to the number of unmanipulated embryos that also resulted in a loss (4/65, 3%; $p = 1.000$).

Those FSL-A manipulated embryos that lead to viable fetuses resulted in a mean fetal weight of 1.0 g (SD±0.1) and a mean placental weight of 0.10 g (SD±0.01). Both values were comparable to those resulting from unmanipulated embryos (1.0 g, SD±0.1; 0.11 g, SD±0.02; $p \geq 0.821$). Gross morphology and development were also comparable.

Table 36 Outcomes of FSL-A manipulated embryos within the Optimal Model. Total numbers of implants, viable fetuses, non-viable fetuses and losses; and the mean fetal and placental weights on day-17 pc. Data collated from 13 recipient mice that each received five FSL-A manipulated and five unmanipulated embryos (media alone). All outcomes were normal.

		Value following transfer		
	Unit	FSL-A	Media alone	P-value
Embryos transferred	n	65	65	-
Implants (%) viable + non-viable fetuses	n	62 (95)	62 (95)	0.717
Viable fetuses (%)	n	48 (74)	44 (68)	0.492
Non-viable fetuses (%)	n	14 (22)	18 (28)	0.041
Losses (%)	n	3 (4)	3 (4)	1.000
Mean fetal weight (±SD)	g	1.0 (±0.1)	1.0 (±0.1)	0.821
Mean placental weight (±SD)	g	0.10 (±0.01)	0.11 (±0.02)	0.895
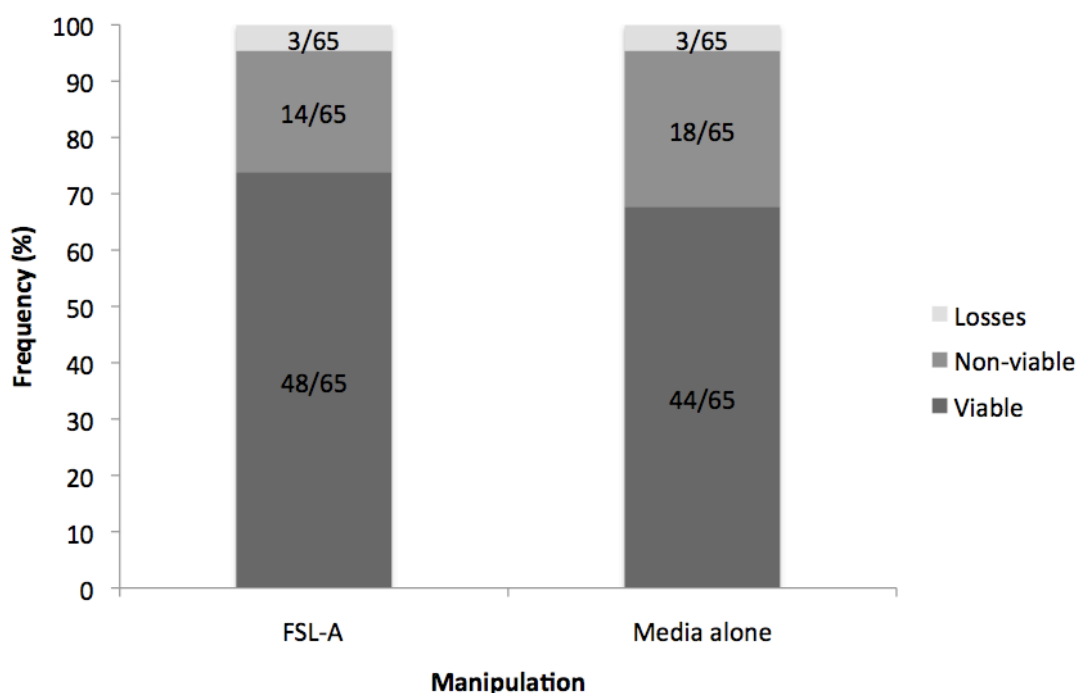
Figure 57 Percentage frequency outcomes of FSL-A manipulated embryos within the Optimal Model. Percentage frequency of viable fetuses, non-viable fetuses and losses on day-17 pc. Values within bars are the number of embryos resulting in that outcome expressed over the total transferred. Numbers of viable + non-viable fetuses equate to the numbers of implants. Data collated from 13 recipient mice that both FSL-A manipulated and unmanipulated embryos (media alone). All outcomes were normal.

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4.5.2.3   Interpretation

No detrimental impact was observed following the transfer of FSL-A modified embryos into the Optimal Model. Therefore, FSL-A was unable to confirm the Optimal Model's ability to detect negative consequences of manipulated embryos.

4.5.3 FSL-Le^Y manipulated embryos

The lewis molecule, Le^Y (Galα1-3[Fucα1-2]Galβ1-4[Fucα1-3]GlcNAc) is an oligosaccharide crucial to murine implantation. It's expression is E and P regulated, and has been detected in endometrial epithelium and embryonic trophectoderm (Kimber & Lindenburg, 1990; Kimber, Lindenburg, & Lundblad, 1988; Zhu, Fenderson, & Du, 1995a; Zhu, Kojima, Stroud, Hakomori, & Fenderson, 1995b; Aplin, 1999). At the time of apposition, the first stage of implantation, Le^Y expression increases dramatically within embryonic trophectoderm (Zhu, et al., 1995b; Aplin, 1999). Such an increase is mirrored by an increase in H-type-1 expression, a ligand of Le^y, on the extra cellular matrix (ECM) component MUC-1 (Aplin, 1999).
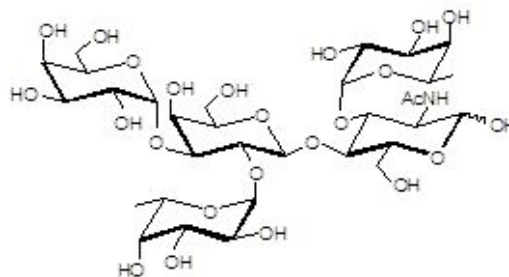
Figure 58 Schematic of Lewis Y antigen.

Interestingly, Carter (2007b) demonstrated a lack of Le^Y expression within *in vitro* cultured murine embryos, when donor retrieval was prior to day-3.5 pc. Carter (2007b) postulated that this lack of Le^Y expression might have contributed towards the low implantation rates seen in human IVF cycles. Carter's (2007b) theory was supported by the dramatic reduction in implantation rates observed following pre-treatment of transferred blastocyst embryos with AH6, a Le^Y antibody (Wang, et al., 1998). Subsequent FSL-Le^Y manipulation restored Le^Y expression, with no detrimental effect on hatching rates (Table 37; Carter, 2007b). However, the impact of FSL-Le^Y on *in vivo* implantation rates is unknown. Therefore, FSL-Le^Y manipulated embryos offered an opportunity to assess the Optimal Model's ability to detect negative consequences. Within this section, donor embryos were incubated with FSL-Le^Y

at 1 mg/mL for two hours. Working concentrations and incubation times were based upon Table 37 (Carter, 2007b).

Table 37 Hatching rate following FSL-Le^Y manipulation of embryos within Carter (2007b). Donor embryos were collected from superovulated prepubescent B6CBAF1/J mice on day-1.5 pc (E1.5[-]). Donor embryos were cultured a further two days in 50 µL M16 media microdrops (Sigma-Aldrich, Cat# M7292, bicarbonate buffered) in 5% CO_2, at 37°C. Donor E3.5(-) embryos were incubated for 2 hours in 1 mg/mL FSL-Le^Y or media alone (M16 media). Following treatment, embryos were incubated for a further twenty-four and forty-eight hours. Numbers of hatching (HgB) and hatched (HdB) blastocysts were counted.

	Treatment		P-value
	FSL-Le^Y	Media alone	
Numbers of embryos treated	41	43	-
Numbers of HgB[a] after 24 hours (%)	22 (55)	25 (60)	> 0.05[b]
Numbers of HgB[a] after 48 hours (%)	36 (85)	37 (86)	> 0.05[b]
Numbers of HdB[a] after 48 hours (%)	9 (21)	5 (13)	> 0.05[b]

a = Grading assigned per Section 3.2.8

b = no significant difference calculated by Carter (2007b).

4.5.3.1 Methodology overview:

Donor embryos were retrieved from pre-pubescent donor B6CBAF1/J mice on day-1.5 pc (Section 3.2.7). Embryos were then *in vitro* cultured in 5% CO_2 at 37°C for two days. Unrestricted-coitus with vasectomised CBA/J male mice, of proven sterility, was used to generate pseudo-pregnant B6CBAF1/J recipient mice (Section 3.2.2 and 3.2.3). Restricted-coitus was not utilized as the specialised cages required had not yet been introduced at the time of experimentation.

On the same day as ET, E3.5(-) embryos were either treated with 1 mg/mL FSL-Le^Y or with culture media alone for two hours (Sections 3.1.2.3 and 3.2.9, Figure 17). Five FSL- Le^Y manipulated embryos were transferred into one uterine horn and five unmanipulated embryos (media alone) into the other uterine horn of each recipient mouse. Computer generated randomised lists assigned the selection of embryos, the uterine horn and the order in which groups were transferred. Based on Figure 17, a total of 11 replicates (recipient mice) were completed over five experimental series (five weeks).

Recipient mice were assessed for ET outcomes day-17 pc (Section 3.2.11). The numbers of viable and non-viable fetuses, and losses were recorded. Photos were taken of intact uteri, followed by the excised fetuses and their corresponding placentae. Each fetus and placenta was weighed, and gross morphology and development were assessed visually. Binary logistic regression and general linear modelling were used to confirm any significant differences between FSL- Le^Y manipulated and unmanipulated embryos. Standard deviation was used to indicate mouse-to-mouse variation in fetal and placental weights.

4.5.3.2 Results:

As seen in Table 38 and Figure 60, all of the outcomes associated with the transfer of FSL-Le^Y manipulated embryos were comparable to those associated with unmanipulated embryos (media alone). Of the 55 FSL-Le^Y manipulated embryos transferred, 49 (89%) resulted in an implant, which was comparable to those resulting from unmanipulated embryos (53/55, 96%; p = 0.811). Of those implants, 45 were viable (82%) and 4 were non-viable (7%), which again were comparable to those resulting from unmanipulated embryos (46/55, 84%; 7/55, 13%; p ≥ 0.541). The remainder of FSL-Le^Y manipulated embryos transferred resulted in a loss (6/55, 11%), which was comparable to the number of unmanipulated embryos also resulting in a loss (2/55, 4%; p = 0.811).

The mean fetal weights (1.0 g, SD±0.1) were comparable to those resulting from unmanipulated embryos (1.0 g, SD±0.08; p = 0.650). In contrast, there was a small but significant difference in mean placental weights (FSL-Le^Y manipulated, 0.11 g, SD±0.01; unmanipulated, 0.12 g, SD±0.02; p = 0.035). Furthermore, gross morphology and development of fetuses resulting from both FSL-Le^Y manipulated and unmanipulated embryos were comparable (Figure 59).

Table 38 Outcomes of FSL-Le^Y manipulated embryos within the Optimal Model. Total numbers of implants, viable fetuses, non-viable fetuses and losses; and the mean fetal and placental weights on day-17 pc. Data collated from 11 recipient mice that received both five FSL-Le^Y manipulated and five unmanipulated embryos (media alone). All outcomes were normal.

		Value following transfer		
	Unit	FSL-Le^Y	Media Alone	P-value
Embryos transferred	n	55	55	-
Implants (%) viable + non-viable fetuses	n	49 (89)	53 (96)	0.811
Viable fetuses (%)	n	45 (82)	46 (84)	0.541
Non-viable fetuses (%)	n	4 (7)	7 (13)	0.562
Losses (%)	n	6 (11)	2 (4)	0.811
Mean fetal weight (±SD)	g	1.0 (±0.1)	1.0 (±0.08)	0.650
Mean placental weight (±SD)	g	0.11 (±0.01)	0.12 (±0.02)	0.035

Unmanipulated   FSL-Le^Y manipulated
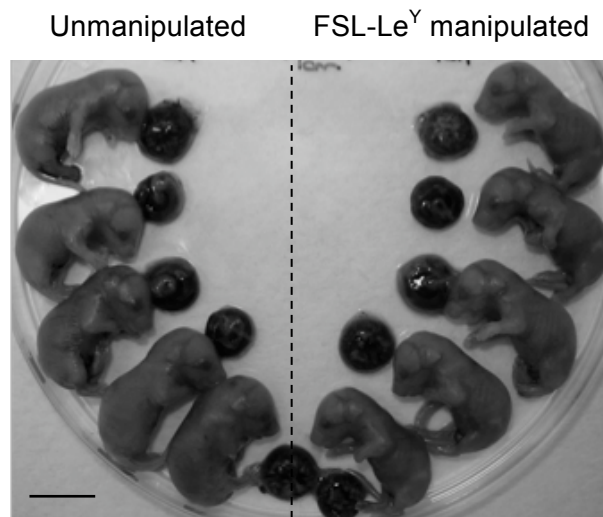
Figure 59 An example of gross morphology and development day-17 pc resulting from FSL-Le^Y manipulated and unmanipulated embryos within the Optimal Model. Horizontal scale bar is 1 cm. Layout as per Figure 27. All fetuses and placentae normal for gestation age.
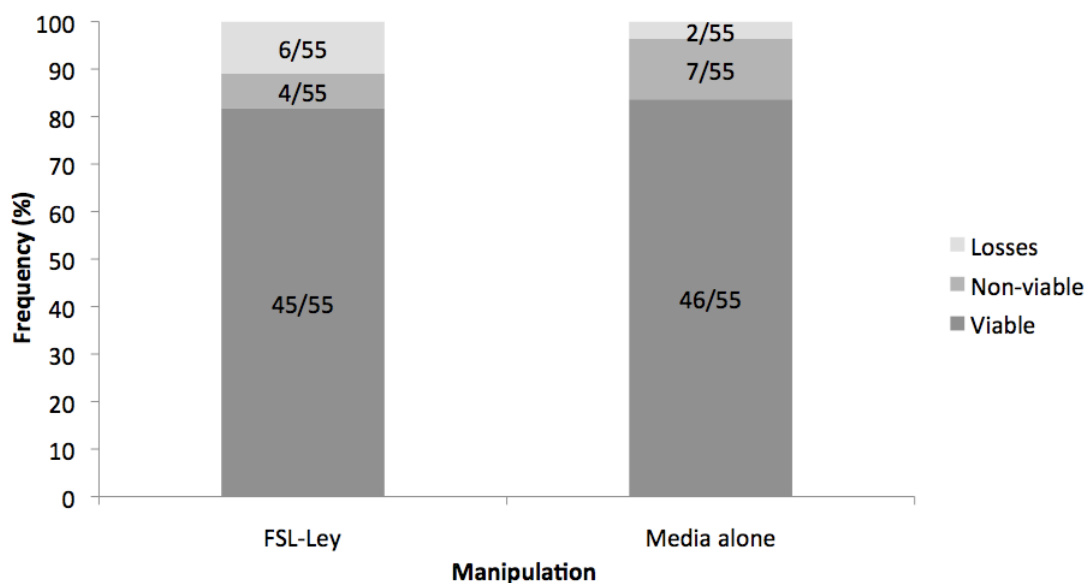
Figure 60 Percentage frequency outcomes of FSL-Le^Y manipulated embryos within the Optimal Model. Percentage frequencies of viable fetuses, non-viable fetuses and losses on day-17 pc. Features as per Figure 57. All outcomes were normal.

4.5.3.3 Interpretation:

The transfer of FSL-Le^Y modified embryos into the Optimal Model resulted in normal outcomes, except for a reduced mean placental weight (0.12 to 0.11 g). Although the reduction was significant, a mean of 0.11 g was still considered normal when compared to Optimal Model's baseline data (0.1 g, SD ±0.02; Table 26). For that reason FSL-Le^Y was considered unsuitable to confirm the Optimal Model's ability to detect negative consequences of manipulated embryos.

4.5.4 FSL-HA manipulated embryos

Hyaluronan (HA) is an omnipresent biological molecule with multiple roles. These include, but are not limited to, water homeostasis, lubrication, and cell-to-cell and cell-to-matrix adhesion (Fraser, Laurent, & Laurent, 1997; Knudson, C., & Knudson, W., 1993; Turley & Moore, 1984). A major component of ECM, HA interacts with proteoglycans contributing to the lattice-like structure (Fraser, et al., 1997; Laurent & Fraser, 1992; Ogston & Stanier, 1953; Turley & Moore, 1984). Expression of HA is widespread throughout the reproductive tract; however, it's expression increases sharply within endometrial stroma at the time of receptivity and implantation

(Afify, Craig, & Paulino, 2006; Carson, Dutt, & Tang, 1987). For this reason, it has been postulated that HA has a role in implantation. This role could be mediated through HAs interaction with CD44, a cell surface glycoprotein expressed on pre-implantation embryonic trophectoderm (Behzad, Seif, Campbell, & Aplin, 1994; Campbell, et al., 1995; Kennel, Lankford, Foote, Shinpock, & Stringer, 1993; Lu, Tian, O'Neill, & King, 2002).

The work of Gardner, Rodrieaez-Martinez, and Lane (1999) brought about the commercial introduction of HA-containing transfer medium. Gardner et al. (1999) was able to demonstrate a significant improvement in murine *in vivo* implantation rates when free-HA containing transfer media was utilised. However, the inclusion of HA in embryo transfer media has been somewhat disputed, where associated implantation rates have been variable (Loutradi, et al., 2007; Loutradi, Tarlatzi, Kolibianakis, & Tarlatzis, 2008; Mahani & Davar, 2007; Simon, et al., 2003; Valojerdi, et al., 2006). As yet, limited numbers of randomised controlled trials have been completed, and those studies that have shown some effect were often funded by commercial businesses. Nonetheless, Williams (2008) was able to significantly improve murine embryo adhesion to *in vitro* human endometrial cell lines, following their manipulation with FSL-HA$_{high}$ and FSL-HA$_{mid}$ (Table 39).

Each molecule shared the foundational FSL-HA structure demonstrated in Figure 61. FSL-HA$_{high}$ had an approximate molecular weight of 17 kDa and consisted of 22 – 100 saccharides. In contrast, the molecular weight of FSL-HA$_{mid}$ was approximately 8 kDa and consisted of 30 – 40 saccharides. Within Williams (2008), FSL-HA$_{mid}$ demonstrated no detrimental effect on murine embryo grade (Table 40). However, increased concentration and incubation times of FSL-HA$_{high}$ had a detrimental effect on blastomere numbers within murine embryos (Table 41; Williams, 2008). Furthermore, the impact of either molecule *in vivo* was unknown. Such data presented a real opportunity to assess the Optimal Model's ability to detect negative consequences of manipulated embryos. As per Table 42, the working concentration of each FSL-HA molecule and the length of incubation were based on Williams (2008).

Table 39 Numbers of FSL-HA$_{mid}$ and FSL-HA$_{high}$ manipulated embryos attached to RL95-2 human endometrial epithelial monolayers within Williams (2008). Donor embryos were collected from superovulated prepubescent B6CBAF1/J mice on day-1.5 pc (E1.5) and *in vitro* cultured in 50 µL EmbryoAssist™ and subsequently BlastAssist™ media microdrops in 5% CO_2, at 37°C. On day-4.5 pc (E4.5) embryos zona pellucida were removed using 5% pronase (in M2 media, Sigma-Aldrich, Cat# M7167, hepes buffered). Embryos were manipulated with 1 mg/mL FSL-HA$_{mid}$ and 2 mg/mL FSL-HA$_{high}$ for 120 minutes in 5% CO_2, at 37°C (1:1 molar concentration, diluent BlastAssist™ media). Embryos were co-cultured with RL95-2 monolayers in 96-well culture plates for 30 minutes in 5% CO_2, at 37°C. Numbers of attached embryos were recorded under inverted microscope.

	Treatment		
	FSL-HA$_{mid}$	FSL-HA$_{high}$	Media alone
Numbers of embryos attached to monolayer (%)	7/14 (50)	11/14 (79)[a]	5/13 (38)[a]

a = significant difference calculated by Williams (2008; $p = 0.021$)
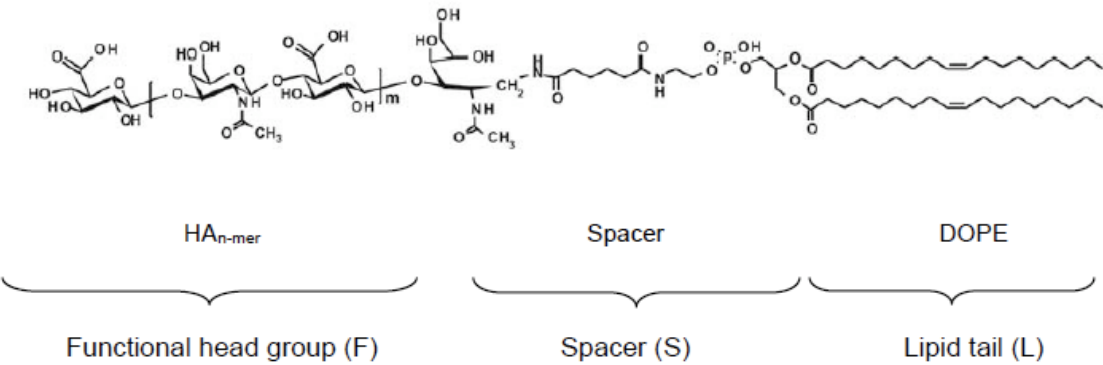
Figure 61 Schematic of a FSL-HA construct. The abbreviation n-mer described how many repeating disaccharide units of **β**1-3 D-N-acetylglucosamine, and **β**1-4 D-glucuronic acid, were contained within each FSL-HA construct. FSL-HA$_{high}$ consisted of 22 – 100 saccharides. FSL-HA$_{mid}$ consisted of 30 – 40 saccharides. Figure sourced from Williams (2008).

Table 40 Embryo grading at 24 and 48 hours subsequent to FSL-HA$_{mid}$ manipulation of embryos within Williams (2008). Donor embryos were collected from superovulated prepubescent B6CBAF1/J mice on day-1.5 pc (E1.5) and *in vitro* cultured for a further 24 hours in 50 µL EmbryoAssist™ media microdrops in 5% CO_2, at 37°C. On day-2.5 pc (E2.5) embryos were incubated for 18 hours in 2 mg/mL FSL-HA$_{mid}$ or media alone (BlastAssist™ media). Following treatment, embryos were incubated for a further twenty-four and forty-eight hours. Numbers of each embryo grade, as per Section 3.2.8, were counted.

		Treatment			
	Unit	FSL-HA$_{mid}$		Media alone	
Time post-manipulation	hrs	24	48	24	48
Early embryos[a] (%)	n	188 (92)	2 (1)	183 (89)	0 (0)
Expanded blastocysts[b] (%)	n	6 (3)	9 (6)	8 (4)	17 (11)
Hatching blastocysts (%)	n	5 (2)	111 (77)	8 (4)	115 (76)
Hatched blastocysts (%)	n	0 (0)	5 (3)	0 (0)	6 (4)
B and C grade embryos (%)	n	6 (3)	17 (12)	7 (3)	13 (9)
Totals	n	205	144	206	151

a = Included M, 5A, 4A, and 3A embryos.
b = Included 2A and 1A embryos.

Table 41 Blastomere number subsequent to FSL-HA$_{high}$ manipulation of embryos within Williams (2008). Donor embryos were collected from superovulated prepubescent B6CBAF1/J mice on day-1.5 pc (E1.5) and *in vitro* cultured for at least further 24 hours in 50 μL EmbryoAssist™ media microdrops in 5% CO_2, at 37°C. Donor embryos were incubated with FSL-HA$_{mid}$ or media alone (BlastAssist™ media) as per described in the table. Following treatment, embryos were incubated for a further twenty-four in 50 μL BlastAssist™ media microdrops. Donor embryos were fixed, stained with Hoechst, and the numbers of blastomeres counted. P-values calculated by Williams (2008).

FSL-HA$_{high}$ concentration (mg/mL)	Embryo age (days pc)	Incubation length (hr)	Blastomere number (±SD)		P-value	Embryo number (n)
			FSL-HA$_{high}$	Media alone		
1.0	2.5 – 3.5	18	39 (±6.4)	48 (±6.5)	0.001[a]	48
1.0	4.5	2	101 (±9.5)	108 (±9.5)	0.152	29
2.0	3.5	2	38 (±3.9)	56 9 (±5.9)	< 0.001[a]	16

a = FSL-HA$_{high}$ significantly different from media alone (p ≤0.05).

4.5.4.1 Methodology overview:

Day-1.5 pc, donor embryos were retrieved from pre-pubescent donor B6CBAF1/J mice (Section 3.2.7). Embryos were then *in vitro* cultured in 5% CO_2 at 37°C for two days. As per Sections 3.2.2 and 3.2.3, pseudo-pregnant B6CBAF1/J recipient mice were produced by unrestricted-coitus with vasectomised CBA/J male mice of proven sterility. At the time of experimentation, restricted-coitus was not available, as the specialised cages required had not yet been developed.

Two different FSL-HA molecules were used for this set of experiments: FSL-HA$_{high}$ and FSL-HA$_{mid}$ (Table 42). On the same day as ET, E3.5(-) embryos were either treated with 1 mg/mL FSL-HA$_{high}$ or 2 mg/mL FSL-HA$_{mid}$ for 18 hours (Sections 3.1.2.3 and 3.2.9, Figure 17). Culture media alone was used as a control for both FSL-HA molecules. At the time of transfer, five manipulated embryos (FSL-HA$_{high}$ or FSL-HA$_{mid}$) were transferred into one uterine horn and five unmanipulated embryos (media alone) into the other uterine horn of each recipient mouse. Computer generated randomised lists assigned the selection of embryos, the uterine horn and the order in which groups were transferred. For those embryos manipulated with FSL-HA$_{high}$, a total of 10 replicates (recipient mice) were completed over three experimental series (3 weeks, Figure 17). For those embryos manipulated with FSL-HA$_{mid}$, 14 replicates (recipient mice) were completed over five experimental series (five weeks, Figure 17).

Seventeen days pc, recipient mice were assessed for ET outcomes (Section 3.2.11). The numbers of viable and non-viable fetuses, and losses were recorded. Photos were taken of intact uteri, followed by the excised fetuses and their corresponding placentae. Each fetus and placenta was weighed, and gross morphology and development were assessed visually. Binary logistic regression and general linear modelling were used to confirm any significant differences between FSL- HA manipulated and unmanipulated embryos. Standard deviation was used to indicate mouse-to-mouse variation in fetal and placental weights.

Table 42 Two different FSL-HA molecules used to manipulate donor embryos. Only one combination was used to treat any one group of embryos. Only one group of manipulated embryos, alongside unmanipulated embryos (media only), were transferred into any one recipient mouse. Concentration and incubation times selected based on the thesis of Williams (2008).

FSL-Molecule	Concentration (mg/mL)	Incubation time (hrs)
HA$_{high}$	1.0	18
HA$_{mid}$	2.0	18

4.5.4.2 Results:

4.5.4.2.1   Consequences of FSL-HA$_{high}$ manipulation of embryos

Data in Table 43 and Figure 63 demonstrate that the numbers of implants following the transfer of FSL-HA$_{high}$ manipulated embryos (42/50, 84%) and unmanipulated embryos (media alone, 43/50, 86%) were comparable ($p = 0.552$). The numbers of embryos that resulted in viable fetuses were comparable between the both FSL-HA$_{high}$ manipulated (31/50, 62%) and unmanipulated embryos (38/50, 76%; $p = 0.172$). The numbers of non-viable fetuses were also comparable following the transfer of FSL-HA$_{high}$ manipulated embryos (11/50, 22%) and unmanipulated embryos (5/50, 10%; $p = 0.162$). In addition, the numbers of FSL-HA$_{high}$ manipulated (8/50, 16%) and unmanipulated (7/50, 14%) embryos that resulted in a loss were comparable ($p = 0.321$).

As demonstrated in Figure 62, fetuses resulting from FSL-HA$_{high}$ manipulated embryos had a small, but significantly reduced mean fetal weight (0.9 g, SD±0.1) in comparison to those resulting from unmanipulated embryos (1.0 g, SD±0.1; $p = 0.009$). In contrast, the mean placental weights resulting from both FSL-HA$_{high}$ manipulated (0.11 g, SD±0.01) and unmanipulated embryos (0.12 g, SD±0.02) were comparable ($p = 0.232$). Gross fetal morphology and development were also comparable (Figure 64).

Table 43 Consequences of FSL-HA$_{high}$ manipulated embryos within the Optimal Model. Total numbers of implants, viable fetuses, non-viable fetuses and losses; and the mean fetal and placental weights on day-17 pc. Data collated from ten recipient mice that each received five FSL-HA$_{high}$ manipulated and five unmanipulated embryos (media alone). The mean fetal weight was significantly reduced (p = 0.009). All other outcomes were normal.

		Value following transfer		
	Unit	FSL-HA$_{high}$	Media Alone	P-value
Embryos transferred	n	50	50	-
Implants (%) viable + non-viable fetuses	n	42 (84)	43 (86)	0.552
Viable fetuses (%)	n	31 (62)	38 (76)	0.172
Non-viable fetuses (%)	n	11 (22)	5 (10)	0.162
Losses (%)	n	8 (16)	7 (14)	0.321
Mean fetal weight (±SD)	g	0.9 (±0.09)	1.0 (±0.1)	0.009[a]
Mean placental weight (±SD)	g	0.11 (±0.01)	0.12 (±0.02)	0.232

a = significantly different p ≤0.05
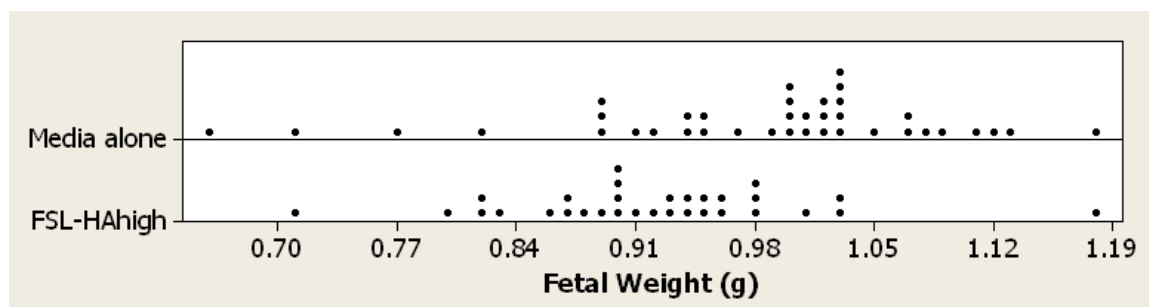
Figure 62 Fetal weights day-17 pc resulting from FSL-HA$_{high}$ manipulated and unmanipulated (media alone) embryos within the Optimal Model. Ten recipient mice, which received five of each group of embryos, contributed to data. Fetal weights resulting from FSL-HA$_{high}$ manipulated embryos were significantly reduced compared to their unmanipulated counterparts (p = 0.009).
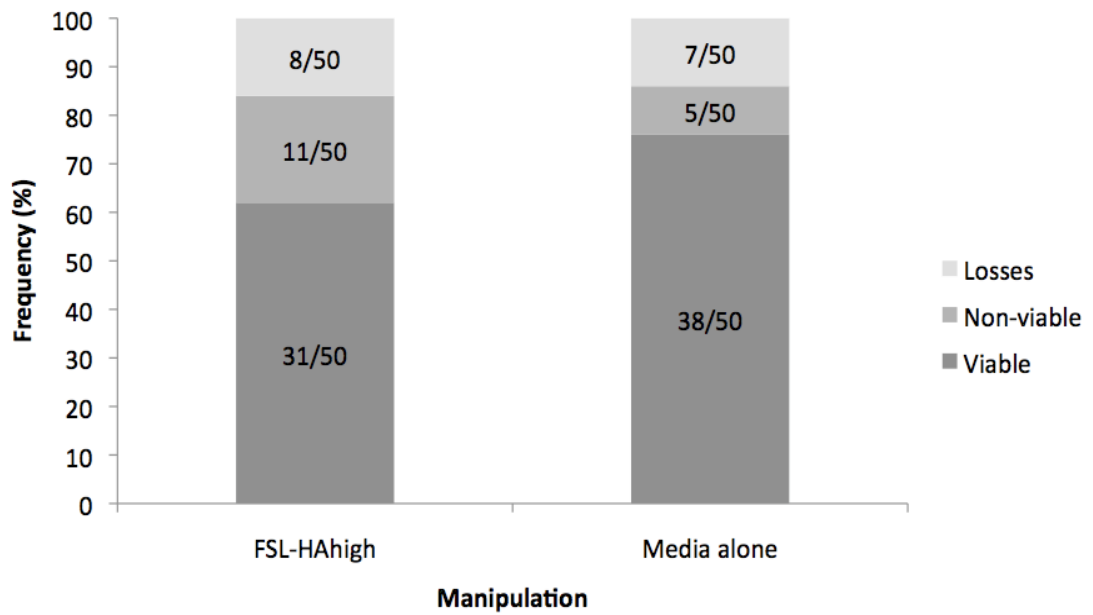
Figure 63 Percentage frequency outcomes of FSL-HA$_{high}$ manipulated embryos within the Optimal Model. Percentage frequencies of viable fetuses, non-viable fetuses and losses day-17 pc. Features as per Figure 57. All outcomes were normal.

Unmanipulated   FSL-HA$_{high}$ manipulated
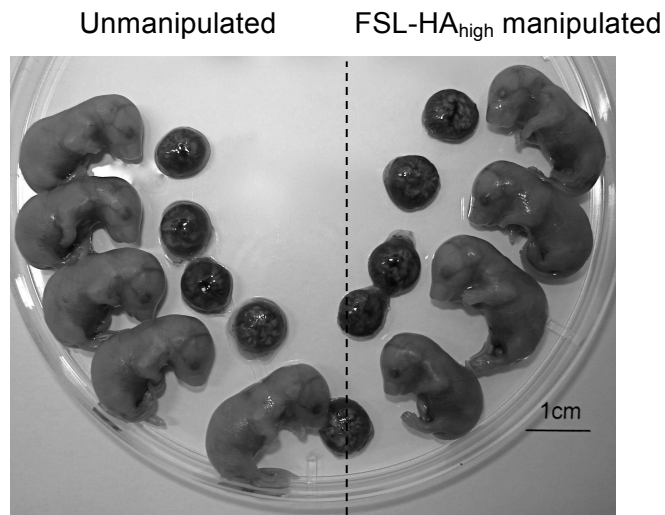
Figure 64 An example of gross morphology and development day-17 pc resulting from FSL-HA$_{high}$ manipulated and unmanipulated embryos within the Optimal Model. All fetuses normal for gestational age. Expected baseline is 9.5 implants per recipient mouse (SD±1.1).

4.5.4.2.2   Consequences of FSL-HA$_{mid}$ manipulation of embryos

Table 44 and Figure 65 demonstrates the comparable outcomes associated with the transfer of FSL-HA$_{mid}$ manipulated embryos and unmanipulated embryos (media alone).   Of the 70 FSL-HA$_{mid}$ manipulated embryos transferred, 62 resulted in an implant (89%).   These numbers of implants were comparable to those resulting from unmanipulated embryos (63/70, 90%; p = 0.402).   Of those implants, 54 resulted in a viable fetus (77%) and 8 resulted in a non-viable fetus (11%), which were also comparable to those resulting from unmanipulated embryos (53/70, 76%; 10/77, 14%; p ≥ 0.154).   The remaining 8 FSL-HA$_{mid}$ manipulated embryos (11%) resulted in a loss, which again was comparable to the number of unmanipulated embryos resulting in a loss (7/70, 10%; p = 0.402).

Both the mean fetal (0.96 g, SD±0.1) and placental (0.11 g, SD±0.02) weights were comparable to their unmanipulated embryo counterparts (0.98 g, SD±0.1; 0.11 g, SD±0.02; p ≥ 0.167).   In addition, fetuses resulting from both FSL-HA$_{mid}$ manipulated and unmanipulated embryos shared comparable gross morphology and development (Figure 66).

Table 44 Outcomes of FSL-HA$_{mid}$ manipulated embryos within the Optimal Model.   Total numbers of implants, viable fetuses, non-viable fetuses and losses; and the mean fetal and placental weights day-17 pc.   Data collated from 14 recipient mice following the transfer of five FSL-HA$_{mid}$ manipulated and five unmanipulated embryos (media alone) into each recipient.   All outcomes were normal.

		Value following transfer		
	Unit	FSL-HA$_{mid}$	Media Alone	P-value
Embryos transferred	n	70	70	-
Implants (%) viable + non-viable fetuses	n	62 (89)	63 (90)	0.402
Viable fetuses (%)	n	54 (77)	53 (76)	0.154
Non-viable fetuses (%)	n	8 (11)	10 (14)	0.321
Losses (%)	n	8 (11)	7 (10)	0.402
Mean fetal weight (±SD)	g	0.96 (±0.1)	0.98 (±0.1)	0.235
Mean placental weight (±SD)	g	0.11 (±0.02)	0.11 (±0.02)	0.167

100
90
80
70
60
50
40
30
20
10
0

Frequency (%)

8/70
8/70
54/70

7/70
10/70
53/70

FSL-HAmid

Media alone

Manipulation

Losses
Non-viable
Viable
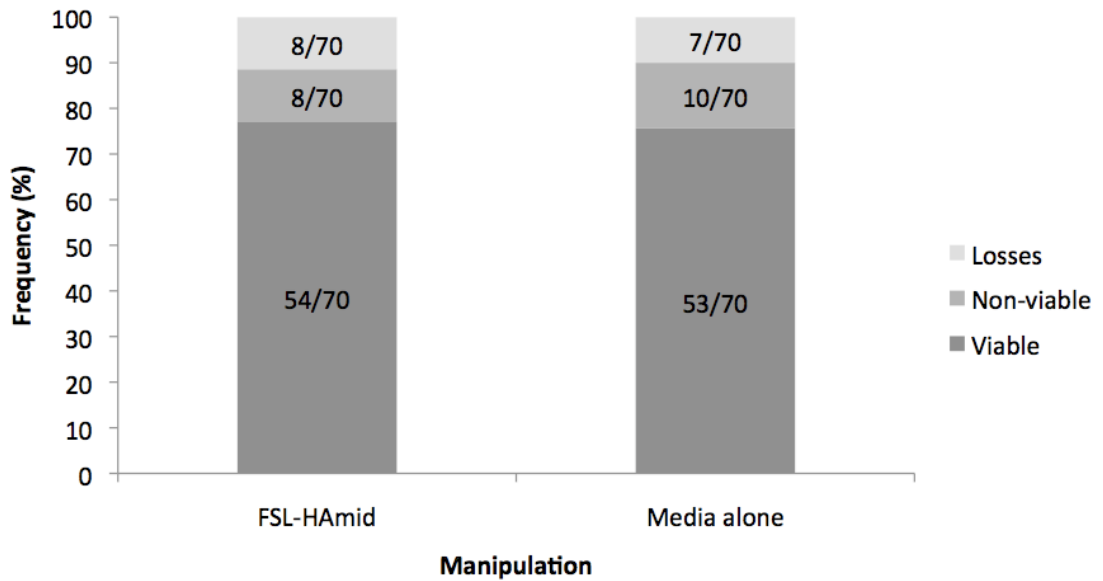
Figure 65 Percentage frequency outcomes of FSL-HA$_{mid}$ manipulated embryos within the Optimal Model. Percentage frequencies of viable, non-viable and losses on day-17 pc. Features as per Figure 57. All outcomes were normal.

Unmanipulated   FSL-HA$_{mid}$ manipulated

1cm
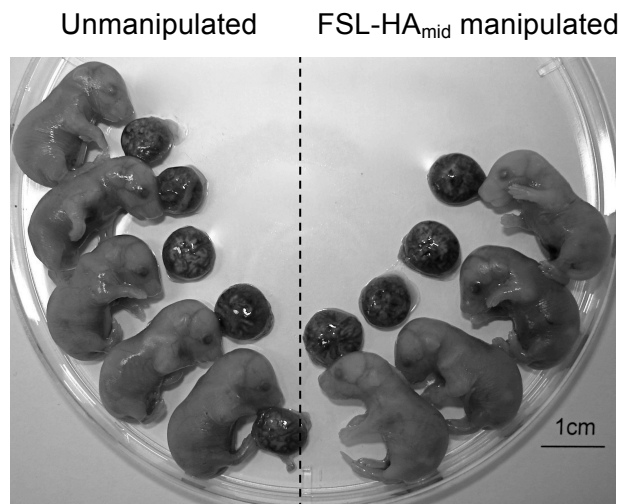
Figure 66 An example of gross morphology and development day-17 pc resulting from FSL-HA$_{mid}$ manipulated and unmanipulated embryos within the Optimal Model. Layout as per Figure 59. All fetuses normal for gestational age. Expected baseline is 9.5 implants per recipient mouse (SD±1.1).

4.5.4.3 Interpretation:

While the majority of outcomes associated with the transfer of FSL-HA$_{high}$ manipulated embryos were normal, a small, but highly significant decrease the mean fetal weight was observed (0.1 to 0.9 g; $p = 0.009$). Reduced mean fetal weight may have correlated with the reduced number of blastomeres observed by Williams (2008) when

donor embryos were also treated with FSL-HA$_{high}$ at 1 mg/mL for 18 hours (Table 41). In contrast, normal outcomes were demonstrated following the transfer of FSL-HA$_{mid}$ manipulated embryos into the Optimal Model. While data indicated that the Optimal Model had the ability to detect negative consequences of manipulated embryos, this project was primarily concerned with implantation rates. Therefore an additional molecule is explored in Section 4.5.5.

4.5.5 BioG manipulated embryos

Up to this point, none of the available FSL-constructs were able to elicit reduced implantation rates and thus confirm the Optimal model's ability to detect negative consequences of embryo manipulation. Compared to FSL-molecules, biotinylated gangliosides (BioG) offered a dramatically different trial molecule as a tool. The ganglioside portion of BioG were naturally occurring glycosphingolipids, containing 20 or more oligosaccharide residues and a sphingolipid tail (Carter, 2007b; Thompson & Tillack, 1985). At the outset, naturally occurring gangliosides are substantially larger than FSL-molecules (Carter, 2007b; Thompson & Tillack, 1985; Williams, 2008). The numbers of oligosaccharides are much greater than those utilised in the functional head group portion of FSL-molecules (Carter, 2007b; Williams, 2008). In addition, the sphingolipid tail typically contains an 18-carbon long-chain base and a 24 fatty acid carbon chain (Bovin et al., 2005a, 2005b as cited in Carter, 2007b). In contrast, the lipid portion of a FSL-molecule lacks the long-chain base, but instead contains two fatty acid chains (Carter, 2007b).

The second point of difference between gangliosides and FSL-molecules was the nature of the working solutions. The source of ganglioside used within this research was extracted from porcine brain tissue, resulting in a heterogeneous mix of gangliosides (Blake, 2003; Bovin et al., 2005a, 2005b as cited in Carter, 2007b). In contrast, the synthetic nature of FSL-molecules allowed for homogenous working molecules (Carter, 2007b). Not only that, but it is also difficult to eliminate the risk of impurities within extractions from animal tissue. In the case of this research, such impurities may act as a tool to detect negative consequences of manipulated embryos.

Biotinylation enabled avidin-labelled molecular detection. However, this research did not focus on labelling systems. While a non-biotinylated version of ganglioside would have been preferable, only BioG was available at the time of research. Nonetheless, BioG manipulated embryos developed normally *in vitro* (Blake, 2003; Carter, 2007b;

Table 45). Yet, there was a lack of clarity surrounding the *in vivo* impact of BioG.
Therefore, BioG offered an opportunity to assess the Optimal Model's ability to detect
negative consequences of manipulated embryos. A working concentration of 5 mg/mL
for 2 hours was selected from the work of Carter (2007b).

Table 45 Development of zona–intact embryos following treatment with BioG within Carter (2007b). Donor embryos were collected from superovulated prepubescent B6CBAF1/J mice on day-1.5 pc (E3.5). Donor embryos were cultured a further two days in 50 µL M16 media microdrops (Sigma-Aldrich, Cat# M7292, bicarbonate buffered) in 5% CO_2, at 37°C. Donor E3.5 embryos were incubated for 2 hours in 5 mg/mL BioG or media alone (M16 media). Following treatment, embryos were incubated for a further twenty-four and forty-eight hours. Numbers of each embryo grade, as per Section 3.2.8, were counted.

	Unit	Treatment			
		BioG		Media alone	
Time post-manipulation	hrs	24	48	24	48
Early embryos[a] (%)	n	0 (0)	0 (0)	0 (0)	0 (0)
Expanded blastocysts[b] (%)	n	17 (35)	0 (0)	23 (50)	3 (7)
Hatching blastocysts (%)	n	31 (65)	40 (83)	23 (50)	37 (80)
Hatched blastocysts (%)	n	0 (0)	8 (17)	0 (0)	6 (13)
Totals	n	48	48	46	46

a = Included M, 5A, 4A, and 3A embryos.
b = Included 2A and 1A embryos.

4.5.5.1   Methodology overview:

As per Section 3.2.7, embryos were produced and collected from pre-pubescent donor
B6CBAF1/J mice on day-1.5 pc. Embryos were then *in vitro* cultured in 5% CO_2
at 37°C for two days. Unrestricted-coitus with vasectomised CBA/J male mice, of
proven sterility, was used to generate pseudo-pregnant B6CBAF1/J recipient mice
(Sections 3.2.2 and 3.2.3). Restricted-coitus was not utilized as the specialised cages
required had not yet been introduced at the time of experimentation.

On the same day as ET, E3.5(-) embryos were either treated with 5 mg/mL BioG or
with culture media alone for two hours (Sections 3.1.2.3 and 3.2.9, Figure 17). Five
BioG manipulated embryos were transferred into one uterine horn and five
unmanipulated embryos (media alone) into the other uterine horn of each recipient

mouse. A total of 18 replicates (recipient mice) were utilized over seven experimental series (seven weeks) as per Figure 17.

Day-17 pc, recipient mice were assessed for ET outcomes (Section 3.2.11). The numbers of viable and non-viable fetuses, and losses were recorded. Photos were taken of intact uteri, followed by the excised fetuses and their corresponding placentae. Each fetus and placenta was weighed, and gross morphology and development were assessed visually. Binary logistic regression and general linear modelling were used to confirm any significant differences between BioG manipulated and unmanipulated embryos. Standard deviation was used to indicate mouse-to-mouse variation in fetal and placental weights.

4.5.5.2 Results:

Table 46 and Figure 67 indicated a reduced number of implants following the transfer of BioG manipulated embryos into the Optimal Model. Of the 90 embryos transferred, 72 (80%) resulted in implants, which was significantly reduced compared to those unmanipulated embryos (media alone, 92%, 83/90; $p = 0.033$). Of those embryos implanted, 64% (58/90) resulted in a viable fetus and 16% (14/90) in a non-viable fetus. Both of these values were comparable to those resulting from unmanipulated embryos (76%, 68/90; 17%, 15/90; $p \geq 0.136$). The remaining 18 embryos resulted in a loss (20%), which was significantly increased compared to those lost unmanipulated embryos (7%, 7/90; $p = 0.033$).

The mean fetal (1.0 g, SD±0.1) and placental (0.11 g, SD±0.01) weights were comparable to those resulting from unmanipulated embryos (0.9 g, SD±0.1; 0.11 g, SD±0.02; $p \geq 0.178$). In addition, BioG manipulated and unmanipulated embryos resulted in comparable gross fetal morphology and development.

Table 46 Outcomes of BioG manipulated embryos within the Optimal Model. Total numbers of implants, viable fetuses, non-viable fetuses and losses; and the mean fetal and placental weights day-17 pc. Eighteen recipient mice were used to collate data. Each received five of each group of embryos. Numbers of implants were significantly reduced, while numbers of losses were significantly increased (p = 0.033). All other outcomes normal.

		Value following transfer		
	Unit	BioG	Media alone	P-value
Embryos transferred	n	90	90	-
Implants (%) viable + non-viable fetuses	n	72 (80)	83 (92)	0.033[a]
Viable fetuses (%)	n	58 (64)	68 (76)	0.136
Non-viable fetuses (%)	n	14 (16)	15 (17)	0.856
Losses (%)	n	18 (20)	7 (7)	0.033[a]
Mean fetal weight (±SD)	g	1.0 (±0.1)	0.9 (±0.1)	0.178
Mean placental weight (±SD)	g	0.11 (±0.01)	0.11 (±0.02)	0.406

a = significantly different p ≤0.05
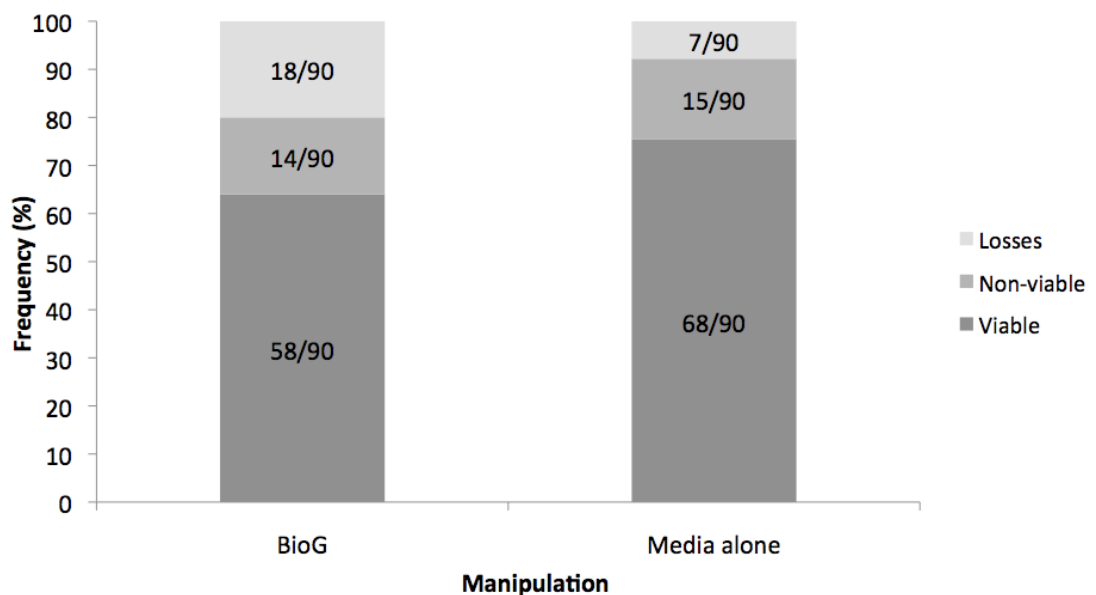
Figure 67 Percentage frequency outcomes of BioG manipulated embryos within the Optimal Model. Percentage frequency of viable fetuses, non-viable fetuses and losses on day-17 pc. Features as per Figure 57. Both groups of embryos were assessed within the same recipient mouse. Data collated from 18 recipient mice. Numbers of implants and losses were significantly different (p = 0.033). All other outcomes were normal.

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4.5.5.3 Interpretation

Subsequent to the transfer of BioG manipulated embryos into the Optimal Model, the percentage frequency of implants was reduced from 92 to 80%. This was mirrored by the increased percentage frequency of losses from 7 to 20%. Furthermore, the percentage frequency of viable fetuses was reduced from 76 to 64%, however, this difference was not statistically significant ($p = 0.136$). Nonetheless, data indicated that the Optimal Model had an ability to detect negative consequences of manipulated embryos. Therefore, overall data from Section 4.5 indicated that the Optimal Model could provide insight into the safety embryo manipulation.

4.6 Ability of Suboptimal Model to detect positive consequences of manipulated embryos

In contrast to the Optimal Model, *in vivo* implantation murine models that exhibited suboptimal implantation rates held the potential to detect positive consequences of manipulated embryos. Prior to non-human primate trials, models identified as suboptimal could indicate whether manipulated embryos held the potential to improve suboptimal implantation rates. As in the Optimal Model, it was important to validate that the Suboptimal Model held an ability to detect positive consequences of manipulated embryos. As stated in Section 4.4, the asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice was selected as the most suitable suboptimal model (Figure 48).

The careful selection of an appropriate molecule, used to manipulate embryos, was of equal importance. It was vital that the selected molecule had the potential to induce a positive outcome following ET. For this reason, molecules trialled within Section 4.5, that were associated with implantation, and resulted in normal ET outcomes, were considered. These included: 1) FSL-Le^Y; 2) FSL-HA$_{high}$; and 3) FSL-HA$_{mid}$. While FSL-Le^Y held a milder impact on viability, the molecule was no longer available for experimentation. The time and expense required to manufacture and examine additional FSL-Le^Y or any other potential molecule exceeded what was available within this research. Nonetheless, FSL-HA$_{high}$ and FSL-HA$_{mid}$ manipulation of embryos resulted in normal ET outcomes and therefore provided an opportunity to explore the Suboptimal Model's potential. Five additional reasons contributed towards HA selection rationale:

1. Hyaluronan holds a potential role in cell-to-cell and cell-to-matrix adhesion (Turley & Moore, 1984).

2. Hyaluronan has also been suggested to have a role in implantation through the receptor-mediated interaction with CD44 (Behzad, Seif, Campbell, & Aplin, 1994; Campbell, et al., 1995; Kennel, Lankford, Foote, Shinpock, & Stringer, 1993; Lu, Tian, O'Neill, & King, 2002).

3. Free-HA containing transfer medium has been demonstrated to significantly improve murine *in vivo* implantation rates (Gardner, et al., 1999).

4. Inserted-HA has also been demonstrated to significantly improve murine embryo adhesion to *in vitro* human endometrial cell lines (Williams, 2008).

5. A number of IVF clinics routinely transfer human embryos in free-HA containing transfer medium in the hope higher implantation rates are facilitated.

For that reason, both inserted and free-HA were used to determine the Suboptimal Model's ability to detect positive consequences of manipulate embryos.

4.6.1 FSL-HA$_{high}$ and free-HA within the Suboptimal Model

Within Section 4.6.1, FSL-HA$_{high}$ (inserted-HA) and free-HA were used to assess the Suboptimal Model's ability to detect positive outcomes of manipulated embryos. Section 4.5.4 indicated that at 1 mg/mL for up to 19 hours, FSL-HA$_{high}$ manipulation of embryos had a detrimental effect on mean fetal weight. However, the *in vitro* work of Williams (2008) suggested that when the length of incubation was reduced to two hours, numbers of blastomeres within murine embryos were normal. For that reason, within this section donor embryos were manipulated with FSL-HA$_{high}$ at 1 mg/mL for no more than two hours.

In an attempt to closely adhere to clinical practice, UTM™ transfer media (Cat# 11520010; Origio Medicult Media, Knardrupvej 2, 2760 Mâlov, Denmark), a commercially available free-HA containing IVF transfer medium, was selected as the free-HA source to transfer unmanipulated embryos (control). In other words, unmanipulated embryos, which were previously cultured in media alone, were transferred into recipient mice with UTM™ (Figure 68). The maximum length of time unmanipulated embryos were incubated in UTM™ prior to transfer was 30 minutes. In contrast, embryos that were manipulated with FSL-HA$_{high}$ for two hours at 1 mg/mL,

were transferred in embryo culture media (media alone) rather than UTM™. Both groups of embryos were compared within the same recipient mouse.
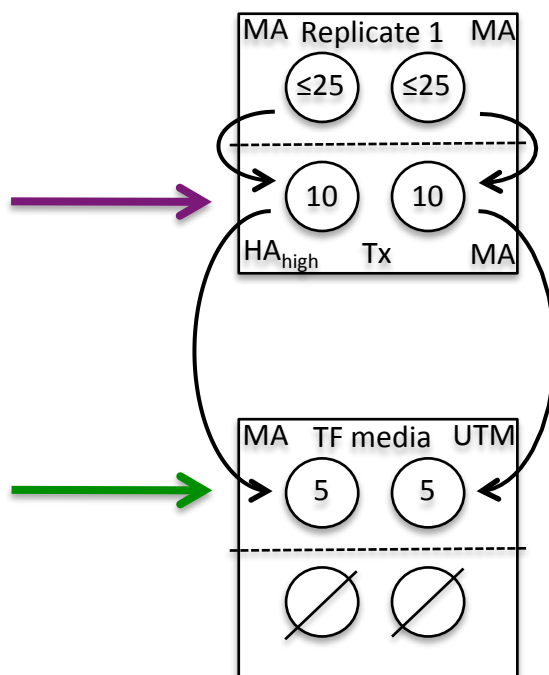
Figure 68 Schematic illustrating embryo culture and transfer media for FSL-HA$_{high}$ and UTM™ treated embryos. On the day of ET, donor embryos were incubated for two hours with FSL-HA$_{high}$ (1 mg/mL), or media alone (purple arrow). Immediately prior to ET, donor embryos were placed in their transfer medium (green arrow). Media alone was to transfer FSL-HA$_{high}$ manipulated embryos. UTM™, a commercially available transfer media containing free-HA, was used to transfer unmanipulated embryos (previously cultured in media alone). Numbers within circles indicated embryo number per 50 µL microdrops. Key: MA, media alone; HA$_{high}$, FSL-HA$_{high}$; Tx, treatment; TF, transfer; UTM, UTM™ transfer media.

As in Section 4.5, donor embryos received the treatment rather than the recipient. For that reason, data was expressed as a percentage of the total number of embryos transferred rather than mean numbers per recipient mouse (percentage implantation frequency). Therefore, the baseline of the Suboptimal Model (6.2 implants per recipient mouse) was converted to a percentage (62%, 56 implants / 90 embryos transferred, Table 32). In this format the baseline could be compared with data from this section.

4.6.1.1 Methodology overview:

Embryo retrieval from pre-pubescent donor B6CBAF1/J mice was performed on day-1.5 pc (Section 3.2.7). Embryos were then *in vitro* cultured in 5% CO$_2$ at 37°C for

three days. As per (Sections 3.2.2 and 3.2.3), pseudo-pregnant B6CBAF1/J recipient mice were produced by unrestricted-coitus with vasectomised CBA/J male mice of proven sterility. Restricted-coitus was not available at the time of experimentation as the specialised cages required had not yet been introduced.

On the same day as ET, E4.5(-) embryos were either treated with 1 mg/mL FSL-HA$_{high}$ or with culture media alone for two hours (Sections 3.1.2.3 and 3.2.9, Figure 17). Five FSL-HA$_{high}$ manipulated embryos were transferred into one uterine horn and five unmanipulated (media alone) embryos into the other uterine horn of R3.5 recipient mice. FSL-HA$_{high}$ manipulated embryos were transferred in embryo culture media and unmanipulated embryos were transferred using UTM™ transfer media. The selection of embryos, the uterine horn and the order in which groups were transferred was randomised to remove bias. A total of eight replicates (recipient mice), produced over three experimental series (three weeks) were completed (Figure 17).

Nine days pc, recipient mice were euthanased, and their uteri excised (Section 3.2.11). The numbers of implants and losses were recorded. Binary logistic regression and general linear modelling were used to confirm any significant differences between FSL-HA$_{high}$ and free-HA manipulated and unmanipulated embryos. Standard deviation was used to indicate mouse-to-mouse variation in fetal and placental weights. In addition, Chi-square tests were used to compare data with the baseline of the Suboptimal Model (62%, Table 32).

4.6.1.2 Results:

Within the Suboptimal Model, FSL-HA$_{high}$ and UTM™ transfer media resulted in comparable ET outcomes (Table 47). Of the 40 FSL-HA$_{high}$ manipulated embryos transferred, 25 (63%) resulted in an implant. This value was comparable to those unmanipulated embryos transferred in UTM™ transfer media (28/40, 70%; p = 0.282). The remaining 15 FSL-HA$_{high}$ manipulated embryos (38%) resulted in a loss, which was comparable to the numbers of lost UTM™ transferred unmanipulated embryos (12/40, 30%; p = 0.282). When implantation frequencies were compared with the full capacity of the Suboptimal Model (62%), neither FSL-HA$_{high}$ manipulated embryos (63%) or UTM™ transferred unmanipulated embryos (70%) resulted in significantly increased implantation frequencies (p ≥ 0.392).

Table 47 Comparison of FSL-HA$_{high}$ and UTM™ transfer media within the Suboptimal Model. Total numbers of implants and losses day-17 pc. Data collated from eight recipient mice, which each received both groups of embryos (five per group). Eight recipient mice utilised. No significant differences were observed between embryo groups and the baseline of the Suboptimal Model (62%, Table 32; p ≥ 0.392).

	Number following transfer (%)		
	FSL-HA$_{high}$	UTM™	P-value compared to treatments
Embryos transferred	40	40	-
Implants viable + non-viable fetuses	25 (63)	28 (70)	0.282
Losses	15 (38)	12 (30)	0.282
P-value compared to Suboptimal baseline	0.976	0.392	-

4.6.1.3 Interpretation:

Subsequent to the transfer of FSL-HA$_{high}$ (inserted-HA) manipulated embryos and UTM™ transfer media (free-HA) into the Suboptimal Model, percentage frequency of implants failed to increase (Figure 69). Not only were both groups of embryos comparable to each other (63%; 70%), but they were also comparable to the Suboptimal Model's baseline (62%, Table 32, Section 4.4). In hindsight, a strict comparison between media alone and UTM™ transfer media would have been useful. However, the numbers of implants and losses between those embryos transferred in UTM™ and the Suboptimal model were comparable (p < 0.0001, Chi-square test). Ultimately, neither FSL-HA$_{high}$ nor UTM™ transfer media were able to demonstrate the Suboptimal Model's ability to detect positive consequences of manipulated embryos.
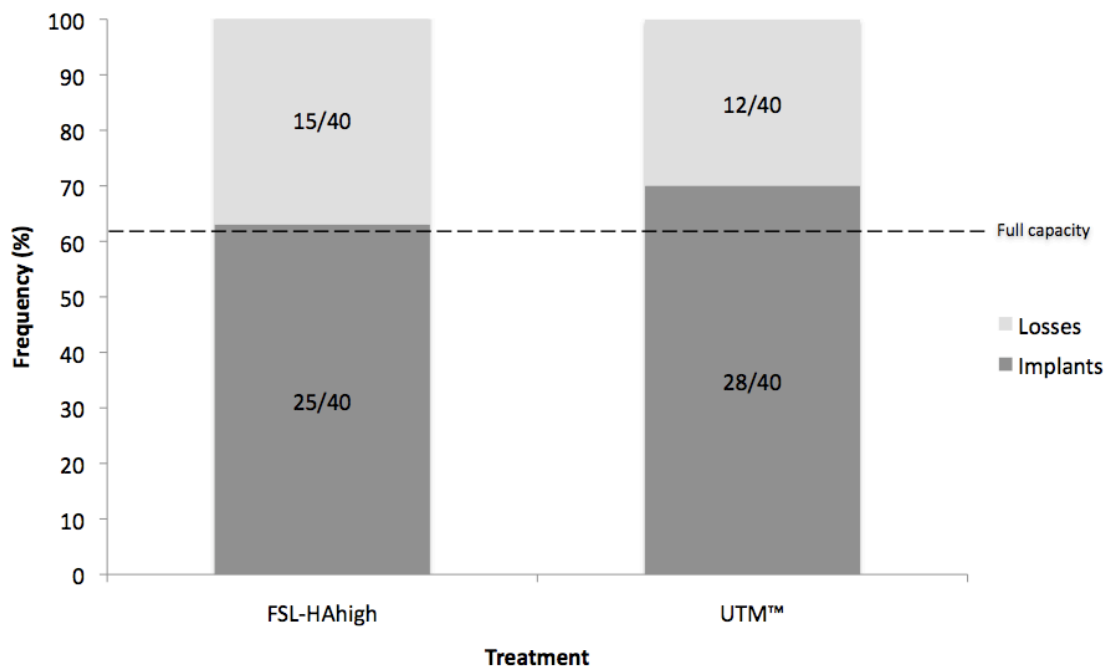
Figure 69 Percentage frequency outcomes day-17 pc of FSL-HA$_{high}$ and UTM™ transfer media within the Suboptimal Model. Data collated from eight recipient mice, which received both groups of embryos (five per group). Values within bars are the numbers of embryos that resulted in the corresponding outcome over the total numbers of embryos transferred. No significant differences between treatments or the baseline of the Suboptimal Model were observed (p ≥ 0.392).

4.6.2 FSL-HA$_{mid}$ manipulated embryos

FSL-HA$_{mid}$ manipulated embryos were the second group of embryos utilised within Section 4.6. At the time Section 4.5 was completed, data that demonstrated successful FSL-HA$_{mid}$ manipulation of embryos in as little as two hours was not available (Williams, 2008). Therefore, within this section FSL-HA$_{mid}$ embryo manipulation would be set at 2 mg/mL for two hours rather than eighteen hours. Manipulated embryos such as these would be transferred into the Suboptimal Model alongside unmanipulated embryos. While embryos transferred in UTM™ transfer media (free-HA) did not result in improved implantation rates, no detrimental effect was observed either (Section 4.6.1). Therefore, to closely adhere to clinical practice, both groups of embryos were transferred in UTM™ transfer within Section 4.6.2.

4.6.2.1 Methodology overview:

Embryos were produced and collected from pre-pubescent donor B6CBAF1/J mice on day-1.5 pc (Section 3.2.7). Embryos were then *in vitro* cultured in 5% CO_2 at 37°C for three days. As per (Sections 3.2.2 and 3.2.3), pseudo-pregnant B6CBAF1/J recipient mice were produced by unrestricted-coitus with vasectomised CBA/J male mice of proven sterility. Restricted-coitus was not available at the time of experimentation, as the specialised cages required had not yet been introduced.

On the same day as ET, E4.5(-) embryos were either treated with 2mg/mL FSL-HA$_{mid}$ or with media alone for two hours (Sections 3.1.2.3 and 3.2.9, Figure 17). Five FSL-HA$_{mid}$ manipulated embryos were transferred into one uterine horn and five unmanipulated (media alone) embryos into the other uterine horn of R3.5 recipient mice. Both groups of embryos were transferred using UTM™ transfer media. Technical bias was minimised by using computer generated randomised lists to assign embryo selection, uterine horn and order groups were transferred in. Based on Figure 17, a total of nine replicates (recipient mice) were completed over four experimental series (four weeks).

Uteri were excised from recipient mice day-9 pc, and the numbers of implants and losses recorded (Section 3.2.11). Binary logistic regression and general linear modelling were used to confirm any significant differences between FSL-HA$_{mid}$ manipulated and unmanipulated embryos. Standard deviation was used to indicate

mouse-to-mouse variation in fetal and placental weights. In addition, Chi-square tests were used to compare data with the baseline of the Suboptimal Model (62%, Table 32).

4.6.2.2 Results:

All outcomes were comparable following the transfer of FSL-HA$_{mid}$ manipulated and unmanipulated E4.5(-) embryos into R3.5 recipient mice. Of the 45 FSL-HA$_{mid}$ manipulated embryos transferred, 21 (47%) resulted in an implant, which was identical to the number of unmanipulated embryos that also resulted in an implant (21/45, 47%; p = 0.880).   The remainder of FSL-HA$_{mid}$ manipulated embryos resulted in a loss (24/45, 53%), which was also identical to the number of lost unmanipulated embryos (24/45, 53%; p = 0.650).   Although the implantation frequencies of both FSL-HA$_{mid}$ manipulated (47%) and unmanipulated embryos (47%) within the Suboptimal Model were reduced compared to the models corresponding baseline, the difference was not significant (62%; p = 0.085).

Table 48 Outcomes of FSL-HA$_{mid}$ manipulated embryos within the Suboptimal Model. Total numbers of implants and losses on day-17 pc, following the transfer of FSL-HA$_{mid}$ manipulated and unmanipulated embryos (media alone) into the Suboptimal Model. Data collated from nine recipient mice. No significant differences between treatments observed.

	Number following transfer (%)		
	FSL-HA$_{mid}$	Media alone	P-value compared to treatments
Embryos transferred	45	45	-
Implants viable + non-viable fetuses	21 (47)	21 (47)	0.880
Losses	24 (53)	24 (53)	0.650
P-value compared to suboptimal baseline	0.085	0.085	-

4.6.2.3 Interpretation:

Following the transfer of FSL-HA$_{mid}$ manipulated and unmanipulated embryos into the Suboptimal Model, 47% of embryos resulted in implants within both groups (Figure 70). Superficially, data suggested that FSL-HA$_{mid}$ manipulated embryos did not demonstrate the Suboptimal Model's ability to detect positive consequences. Yet, when examined

more closely, the implantation frequencies seen in Figure 70 (47%) were reduced in comparison to the baseline (62%). The resulting p-value (0.085) demonstrated a trend towards significance. Therefore, data raised questions whether technical differences between the two scenarios contributed. Two examples of technical differences included: 1) the use of UTM™ as transfer medium; and 2) the use of unrestricted-coitus (Section 4.6) verses restricted-coitus (Suboptimal model; Section 4.4) to generate pseudo-pregnant recipient mice.

Within Section 4.6, unmanipulated embryos were transferred in UTM™ transfer media. In contrast, media alone was used to transfer unmanipulated embryos into the Suboptimal model (Section 4.4). While a strict comparison between embryos transferred in UTM™ or media alone would have been useful, numbers of implants and losses were comparable between the two groups in Section 4.6.2 ($p < 0.001$, Chi-square test). Therefore, the use of UTM™ transfer media was not thought to be responsible for the reduced implantation frequencies observed.

The second possible reason for the reduced implantation frequencies was a difference between unrestricted and restricted-coitus. While both sets of experiments generated pseudo-pregnancy through coitus with vasectomised male mice, the length of time accessible to recipient female mice differed. Within this section, recipient mice underwent unrestricted-coitus, where vasectomised male mice had unlimited access to recipient female mice. In contrast, experiments that determined the Suboptimal Model's baseline utilised recipient mice that underwent restricted-coitus (Section 4.4). In this instance, the length of time vasectomised male mice had access to recipient female mice was limited. As a consequence the window of time in which coitus could occur was reduced compared to those recipient mice that underwent unrestricted-coitus.

The difference in generating pseudo-pregnant recipient mice lay in the point of completion for each section. The Suboptimal model, which acted as a foundation for Section 4.6.2, was originally established prior to the introduction of restricted-coitus. At the time the high implantation frequency seen in the Optimal Model (95%, Table 26), suggested the use of unrestricted-coitus was not a concern. However, mouse-to-mouse variability within models continued to be concerning, and the transfer of E4.5(-) embryos into R3.5 recipient mice was repeated using restricted-coitus (Section 4.4).
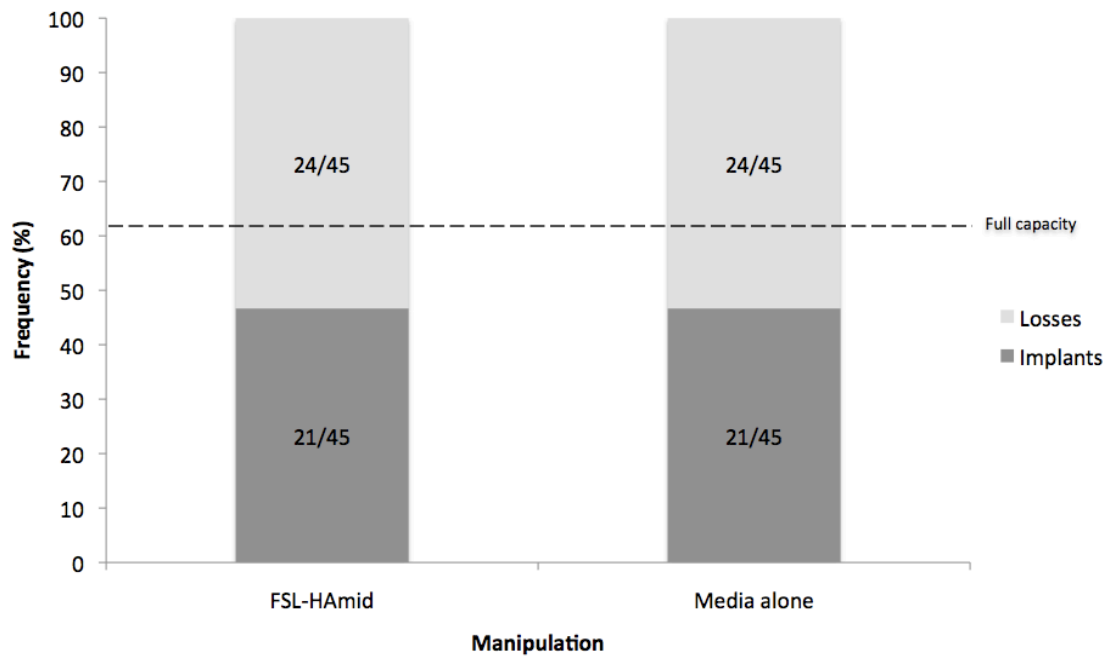
Figure 70 Percentage frequencies day-17 pc of FSL-HA$_{mid}$ manipulated embryos within the Suboptimal Model. Values within bars are the numbers of embryos that resulted in the corresponding outcome over the total numbers of embryos transferred. Data collated from nine recipient mice (five embryos from each group within the same mouse). No significant differences observed.

If unrestricted-coitus were responsible for observed reduced implantation frequencies, the model assessed within this section would differ from the established Suboptimal Model. If held true, data could not be used to assess the Suboptimal Model's ability to detect positive consequences of manipulated embryos. Therefore, Section 4.6.3 compared original (unrestricted-coitus) and final (restricted-coitus) data associated with the Suboptimal model.

4.6.3 Impact of unrestricted-coitus within the Suboptimal Model

The aim of Section 4.6.3 is to determine whether recipient mice that underwent unrestricted-coitus were responsible for the reduced implantation frequencies observed in Section 4.6.2. This was achieved by comparing initial data, which surrounded the transfer unmanipulated E4.5(-) embryos into R3.5 recipient mice that had undergone unrestricted-coitus, with the final Suboptimal Model, which utilised recipient mice that had undergone restricted-coitus (Section 4.4). The mean number of implants per recipient mouse was compared rather than implantation frequency, as differing variables were between recipient mouse rather than donor embryos.

4.6.3.1 Methodology overview:

Embryo retrieval from pre-pubescent donor B6CBAF1/J mice was performed on day-1.5 pc (Section 3.2.7). Embryos were then *in vitro* cultured in 5% CO_2 at 37°C for three days. As per (Sections 3.2.2 and 3.2.3), pseudo-pregnant B6CBAF1/J recipient mice were produced by unrestricted-coitus with vasectomised CBA/J male mice of proven sterility. Unmanipulated E4.5(-) embryos (five per uterine horn) were transferred into eight R3.5 recipient mice. Embryo culture media was used for transfer.

Ten days pc, female mice were euthanased, their uteri excised and the mean numbers of implants and losses were recorded (Section 3.2.11). Outcomes were compared with the asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice that had undergone restricted-coitus (Table 32, Section 4.4). Fisher's exact tests were used to analyse data.

4.6.3.2 Results:

A mean implantation rate of 6.3 implants per recipient mouse (SD±2.9) resulted following the transfer of E4.5(-) embryos into R3.5 recipient mice that had undergone unrestricted-coitus (Table 49). This value was comparable to their recipient counterparts that had undergone restricted coitus (6.2, SD±2.6; p = 1.0). Not only that, but the range of implants within individual recipient mice (2 – 10 implants; Figure 71) was identical to those recipients that experienced restricted-coitus (2 – 10 implants). All transferred embryos were A-grade quality and of normal developmental stage for E4.5(-) embryos (Table 49). The remainder of embryos resulted in mean numbers of losses (3.8, SD±2.9) comparable to their restricted-coitus counterparts (3.8, SD±2.6; p = 1.0).

Table 49 Impact of unrestricted-coitus on mean outcomes within the Suboptimal Model. Mean numbers of implants and losses on day-10 pc, following the transfer of E4.5(-) embryos (five per uterine horn) into eight R3.5 recipient mice (unrestricted-coitus). No significant differences between recipient mice that underwent unrestricted and restricted-coitus were observed.

Outcome	Unrestricted-coitus		Restricted-coitus		P-value of means
	Mean per mouse (±SD)	Total	Mean per mouse (±SD)	Total	
Implants viable + non-viable fetuses	6.3 (±2.9)	50	6.2 (±2.6)	56	1.0
Losses	3.8 (±2.9)	30	3.8 (±2.6)	34	1.0
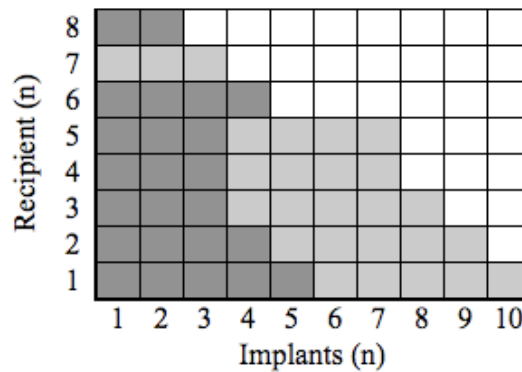
Figure 71 Impact of unrestricted-coitus on numbers and position of implants within the Suboptimal Model. Features as per Figure 20. Data ordered from highest to lowest numbers of implants per recipient mouse. Recipients resulted in 2 – 10 implants each. Mean implantation rate was 6.3 implants per recipient mouse. Data was comparable to restricted-coitus within the Suboptimal Model (2 – 10 implants; 6.2 implants per recipient mouse; p = 0.967).

Table 50 Grade allocation of E4.5(-) embryos prior to transfer into R3.5 recipient mice, which underwent unrestricted-coitus. Each recipient mouse represented one replicate, and was grouped by experimental series (Figure 15). Donor embryos were retrieved from a pool of prepubescent donor mice for each experimental series. Embryo grades, indicating stage of development and quality, were allocated as per Section 3.2.8 (Table 17). Mean numbers of implants were assessed on day-17 pc. Recipients 1 through to 10 represent recipients 8, 6, 5, 4, 7, 1, 2 and 8 in Figure 71. Key: Recip, recipient; Exp, experimental; ET, embryo transfer; 2A – 1A, blastocyst; HgB, hatching; HdB, hatched.

Recip	Exp series	Numbers per recipient mouse				
		Embryo grade at time of ET (%)				Implants
		2A	1A	HgB	HdB	
1	1	1 (10)	5 (50)	4 (40)	-	2
2	1	1 (10)	3 (30)	6 (60)	-	4
3	2	-	1 (10)	9 (90)	-	7
4	2	-	1 (10)	9 (90)	-	7
5	2	1 (10)	-	9 (90)	-	3
6	3	-	1 (10)	9 (90)	-	10
7	3	-	2 (20)	8 (80)	-	9
8	3	-	2 (20)	8 (80)	-	8

4.6.3.3 Interpretation:

The use of unrestricted coitus did not reduce the mean implantation rate following the transfer of unmanipulated E4.5(-) embryos into R3.5 recipient mice. Instead, mean numbers of implants per recipient mouse were comparable between both unrestricted-coitus (6.3) and restricted-coitus (6.2). Therefore, data suggested that the reduced implantation frequencies observed in Section 4.6.2 were not due to the use of recipient mice that underwent unrestricted-coitus. It is worth noting, however, that robustness of data would have been improved if both scenarios were completed at the same time and must be considered when interpreting results. Nonetheless, data suggested conclusions from Sections 4.6.1 and 4.6.2 could be valid. The inability of free-HA to improve implantation rates within the Suboptimal Model was unexpected. Previous research demonstrated improved murine implantation rates following the use of free-HA containing transfer medium in ET (Gardner, et al., 1999). In addition, a recent Cochran systematic review has confirmed a small, but significant increase in human implantation and pregnancy rates as a result of free-HA containing transfer medium (Bontekoe, et al., 2010). Therefore, Section 4.6.4 attempts to repeat original experiments, which had led to the commercial introduction of free-HA containing IVF transfer medium.

4.6.4 Free-HA manipulated embryos

To establish the impact of free-HA within this research, the innovative murine work of Gardner et al. (1999), which contributed to the introduction of the commercially available Embryoglue® media, was repeated. Unmodified E4.5(-) donor embryos were transferred into R3.5 recipient mice (Suboptimal model) using a free-HA containing medium (0.5 mg/mL). However, to match the work of Gardner et al. (1999), the experimental design was altered slightly from the Suboptimal Model (Table 32). These differences included: 1) donor embryos were retrieved one day earlier (day-0.5 pc) and as a consequence, embryos were *in vitro* cultured one day longer; 2) recipient mice were generated using unrestricted-coitus; and 3) ET outcomes were assessed one day earlier (day-16 pc).

4.6.4.1 Methodology overview:

Two-cell embryos were collected from pre-pubescent donor B6CBAF1/J mice on day-0.5 pc (Section 3.2.7). A 300 IU/mL sterile hyaluronidase solution was used to remove the cumulus cells of the 2-cell embryos. Once washed, embryos were *in vitro*

cultured in 5% CO_2 at 37°C until day-3.5 pc. Pseudo-pregnant B6CBAF1/J recipient mice were produced by unrestricted-coitus with vasectomised CBA/J male mice of proven sterility (Sections 3.2.2 and 3.2.3). Restricted-coitus was not utilized as the specialised cages required had not yet been introduced at the time of experimentation. As per Section 4.4, Unmanipulated E4.5(-) embryos (five per uterine horn) were transferred into ten R3.5 recipient mice (Suboptimal Model). Free-HA containing embryo culture media (HMW-HA at 0.5 mg/mL, not UTM™) was used to transfer one group of embryos into one horn, and media alone for the remaining uterine horn (Section 3.1.2.4). A total of ten replicates (recipient mice) were completed over four experimental series (four weeks). On day-16 pc, female mice were euthanased and the mean numbers of implants and losses were recorded (Section 3.2.11). Binary logistic regression was used confirm any significant differences.

4.6.4.2 Results:

As seen in Table 51, the number of embryos that resulted in implants following the use of 0.5 mg/mL Free-HA as transfer media (26/50, 52%), were comparable to those embryos transferred in media alone (28/50, 56%; p = 0.628). The remainder of embryos resulted in a loss (24/50, 48%), which was also comparable to those embryos transferred in media alone (22/50, 44%; p = 0.628).

Table 51 ET outcomes associated with free-HA containing transfer medium. Total numbers of implants and losses following the transfer of unmanipulated E4.5(-) embryos into ten R3.5 recipient mice, using either free-HA containing embryo culture media or media alone (day-16 pc). No significant differences observed.

	Number following transfer (%)		
	Free-HA	Media alone	P-value
Embryos transferred	50	50	-
Implants viable + non-viable fetuses	26 (52)	28 (56)	0.628
Losses	24 (48)	22 (44)	0.628

4.6.4.3 Interpretation:

Free-HA containing embryo culture media (0.5 mg/mL) did not demonstrate any improvement in suboptimal implantation frequencies (Figure 72). Fifty-two percent of embryos transferred in free-HA containing medium resulted in an implant. This percentage frequency was comparable to those transferred in media alone (56%). However, data differed to Gardner et al. (1999), who demonstrated significantly increased implantation rates with free-HA containing transfer media. Thus, this research contributed towards the ongoing debate surrounding HA-containing transfer media, and the weight given to commercially funded research. Notwithstanding, this research indicated that inserted and free-HA were unsuitable to assess the Suboptimal Model's ability to detect positive consequences of manipulated embryos.
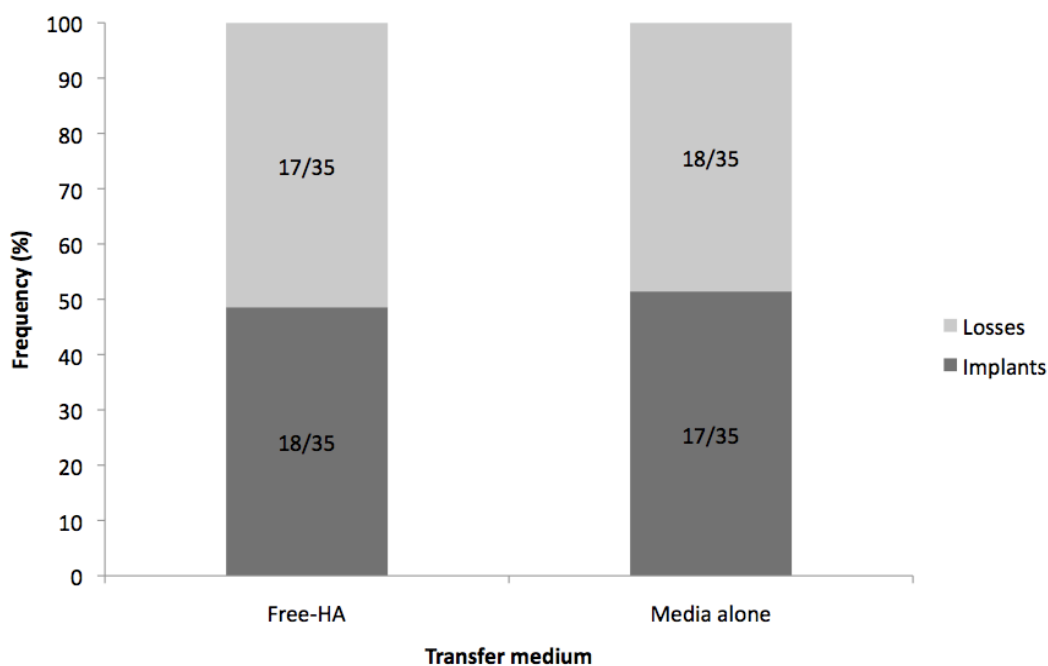
Figure 72 Percentage frequency outcomes associated with Free-HA containing medium in ET. Percentage frequencies of implants and losses following the transfer of unmanipulated E4.5(-) embryos into R3.5 recipient mice, using either Free-HA containing embryo culture media or media alone (day-16 pc). Values within bars are the numbers of embryos resulting in that corresponding outcome over the total numbers of embryos transferred. No significant differences were observed

Due to the lack of potential molecules available, only one group of molecules were used to assess the ability of the Suboptimal Model. The functional head group of FSL-Le[Y] has known involvement with implantation, thus held preference for further exploration. However, FSL-Le[Y] was no longer available. The remaining two molecules trialled within Section 4.5, FSL-A and BioG, were not known to be involved with

implantation. Consequently, each molecule would require additional adhesion systems to improve implantation rates. For example, Carter (2007b) demonstrated increased adhesion of murine embryos with single cell suspensions of murine endometrial epithelial cells *in vitro* (Table 52). Carter treated embryos with BioG, avidin, and BioIgGA to increase their adherence to FSL-A treated endometrial epithelial cells. While data provided proof of principle, it would be difficult to translate this adhesion system to the *in vivo* context. Furthermore, the time required to manufacture and examine additional molecules was beyond the resources of this research. Scope for further research is discussed in Chapter 8.

Table 52 Attachment of BioG/avidin/BioIgGA treated murine embryos with FSL-A treated single cell murine endometrial epithelial cell suspensions within Carter (2007b). Donor embryos collected from superovulated prepubescent B6CBAF1/J mice day-3.5 pc (E3.5). Zona pellucida immediately removed using 5% pronase (in M2 media, Sigma-Aldrich, Cat# M7167, hepes buffered) and incubated for 2 hours in 5 mg/mL BioG (M16, Sigma-Aldrich, Cat# M7292, bicarbonate buffered). Donor embryos treated with 0.1 mg/mL avidin (Sigma-Aldrich, Cat# A-9275), and BioIgGA (Diagnostics, Scotland) at 1:3 dilution, both for 1 hour at 37°C (diluent M2 media). Single endometrial epithelial cell suspensions were incubated with 1 mg/mL of FSL-A (diluent M2 media). Donor embryos and endometrial epithelial cells were treated with SNARF (Molecular Probes®, C-6826) or CMFDA (Molecular Probes®, C2925) fluorescent stains, and incubated together for 30 minutes. Numbers of endometrial epithelial cells attached to embryos were counted under fluorescence microscope.

	Endometrial Cell Treatment	
	FSL-A	Control
Numbers of BioG/avidin/BioIgG[A] treated embryos introduced to assay	6	5
Range of SCEE cells attached	5 – 37	0 – 2
Mean number of SCEE attached	15.7[a]	1.6[a]

a = significant difference calculated by ANOVA in Carter (p = 0.035; 2007b).

5 Results - Refinement of *in vivo* implantation murine models

The investigation of a selection of suboptimal models revealed significant mouse-to-mouse variability, resulting in inconsistent implantation rates. Extensive literature reviews were unable to identify reasons for the observed mouse-to-mouse variability within models. In fact, substantial gaps in knowledge surrounding the beginning of endometrial receptivity and the influence altered embryo-endometrial developmental synchrony had on its timing were identified. For this reason, Chapter 5 investigates the beginning of endometrial receptivity in both natural mouse pregnancy, and synchronous and asynchronous uterine ET. It was hoped that findings would provide an insight into mouse-to-mouse variability, exhibited as inconsistent implantation rates, which were observed throughout this research.

5.1 Exploring the beginning of endometrial receptivity in natural mouse pregnancy

Naturally pregnant mice were assessed for the presence of implants at 88, 90 or 92 hours pc. The mean numbers of implants observed at these times, reflected the mean numbers of embryos that had fully developed and initiated implantation by that time. In contrast, the baseline of natural mouse pregnancy (day-17 pc) reflected the mean numbers of embryos that had fully developed and initiated implantation prior to the end of endometrial receptivity.

Time-points of 88, 90 and 92 hours pc were selected in an attempt to span both the pre-receptive and receptive periods. The literature indicated that the endometrium was receptive by 92 hours pc, therefore, this time-point was selected to incorporate the receptive period (McMaster, Dey, & Andrews, 1993). It was anticipated that time-points as early as 88 hours pc would incorporate the pre-receptive period. Initially, assessing for implants four hours earlier than previously reported may have seemed like a small shift. However, murine endometrium is thought to be receptive for at least 12 hours, but no more than 24 hours long (Doyle, et al., 1963; Ma, et al., 2003; Paria, et al., 1993; Psychoyos, 1973a, 1973b; Yoshinaga, 1988). Therefore, four hours equated up to 33% of the total endometrial receptivity period.

The length of time between assessed time-points was also important to consider. If time-intervals were as close as an hour apart, significant differences could be difficult to differentiate. Whereas, if assessed time-points were more than four – five hours apart, in context of the total length of endometrial receptivity, vital information could be lost. For this reason, two-hourly time-intervals were selected for this research.

Restricted-coitus was used to produce naturally pregnant mice for this study. Specially designed cages limited the time available for coitus (Figure 14). Fertile CBA/J male mice were only allowed access to B6CBAF1/J female mice from the mid-point of the dark phase. The presence of seminal plugs, used to confirm pregnancy, were not checked for another eight to ten hours following the first point of male-female contact. Coitus, which included a number of preliminary mounts prior to ejaculation, could occur at any point during that period of time once female mice had ovulated. For that reason, endometrial development age at the time of assessment could be equal to or less than real-time pc, which was taken from the mid-point of the dark cycle. Nonetheless, only one other study incorporated a type of restricted-breeding into their experimental design, albeit for limited numbers of mice (Potts, 1968). Thus, this research offered improved statistical robustness compared to the literature.

To help decipher the beginning of endometrial receptivity, time-points, 88 , 90 and 92 hours pc, were compared with the baseline of natural pregnancy. If time-points were close to the beginning of endometrial receptivity, the mean numbers of implants would be reduced compared to its baseline. In contrast, those time-points close to the end of endometrial receptivity would result in mean numbers of implants that reflected its baseline. In addition, the intensity of blue-bands, which indicated the extent of vascularisation and implantation, were graded.

It was hoped that data would reveal two things. Firstly, that data would indicate when endometrial receptivity might begin under normal circumstances. Secondly, that data would confirm appropriate experimental design to assess the time of endometrial receptivity within synchronous and asynchronous ET (Section 4.2).

5.1.1   Methodology overview:

Power analysis, using the PASS software programme, determined the need for a minimum of four mice at each assessed time-point. This translated to a minimum of four replicates per assessed time-point, which was completed over five experimental series (five weeks; Figure 18). As per Section 4.1 naturally pregnant B6CBAF1/J

female mice were produced through their restricted-coitus with CBA/J male mice of proven fertility. The presence of seminal plugs the following morning indicated successful coitus and represented day-0.5 pc. Naturally pregnant B6CBAF1/J female mice were then housed individually until the day of autopsy.

On day-3.5 pc, mice were assessed for the presence of implantation at 88, 90 or 92 hours pc (Section 3.2.11). Prior to euthanasia, mice were injected intravenously with Evans Blue dye to allow visualisation of implants. Blue-bands indicated the extent of vascularisation and implantation. Following euthanasia, uteri were excised, and the numbers of implants, indicated by the presence of blue-bands, were recorded. In addition the intensity of each blue-band was graded (Section 3.2.11). In addition to standard deviation, Chi-square tests were used to confirm any significant difference between time-points. Fisher's exact tests were used to compare assessed time-points with the baseline of natural mouse pregnancy.

5.1.2   Results:

Implants were observed at all three time-points (Table 53). Firstly, a mean of 1.8 implants per mouse (SD±2.1) was observed at 88 hours pc, followed by 1.3 implants per mouse (SD±2.5) at 90 hours pc. Finally, a mean of 4.2 implants per mouse (SD±2.4) was observed at 92 hours pc. The mean numbers of implants at 92 hours pc were higher than both 88 and 90 hours pc. Chi-square tests indicated observed differences were significantly different (p = 0.002).

All time-points demonstrated significantly reduced mean numbers of implants compared to the baseline of natural pregnancy (p < 0.001). At least 50% of recipient mice resulted in no implants at 88 and 90 hours pc (Figure 73). At 88 hours pc, two out of four recipient mice resulted in no implants. At 90 hours pc, three out of four recipient mice resulted in no implants. In contrast, 100% of recipient mice resulted in implants at 92 hours pc. Of the total numbers of blue-bands detected (implants), 33% (11/33) were graded as a 1+ and 67% (22/33) were graded as a 2+. No blue-bands were graded as a 3+ (Figure 74).

Table 53 Mean numbers of implants observed at 88, 90 and 92 hours pc within natural mouse pregnancy. Time-points were significantly different from each other and their corresponding baseline (p < 0.002).

Time of autopsy pc (hrs)	Number of mice	Mean number of implants per recipient (±SD)	P-value compared to baseline
88	4	1.8 (±2.1)	< 0.001[a]
90	4	1.3 (±2.5)	< 0.001[a]
92	5	4.2 (±2.4)	< 0.001[a]

a = significantly different p ≤ 0.05
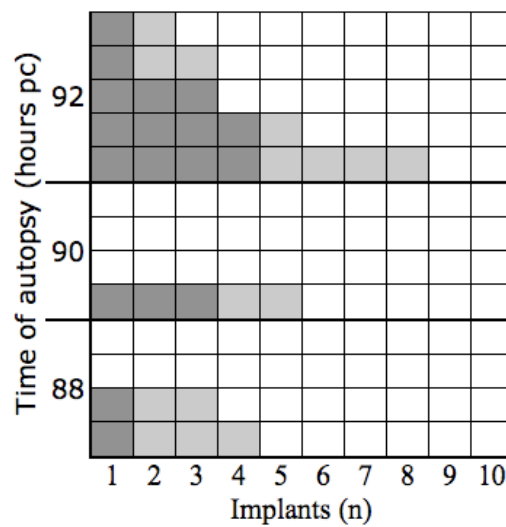
Figure 73 Numbers and location of implants within natural mouse pregnancy at 88, 90 and 92 hours pc. Each row represents one recipient mouse. The numbers of shaded squares indicate the numbers of implants. Location of each implant is indicated by either dark-grey squares (left uterine horn) or light-grey squares (right uterine horn). Five out of the eight recipient mice assessed at 88 and 90 hours pc resulted in no implants.
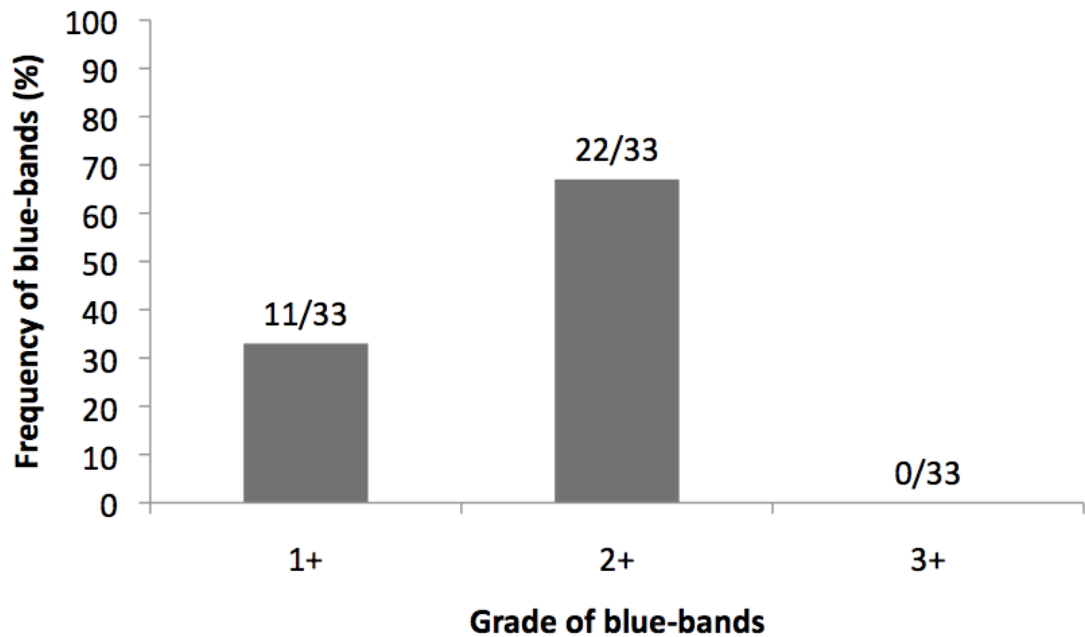
Figure 74 Total frequencies of blue-band grades on day-3.5 pc of natural mouse pregnancy. Data collated 88, 90 and 92 hours pc (day-3.5 pc). Values above bars are the numbers of bands over the total numbers of bands detected. Grades as per Protocol 9.7.

5.1.3 Interpretation:

Implants were detected as early as 88 hours pc, indicating that each time-point was inclusive of the receptive period. Each time-point resulted in reduced mean numbers of implants (1.8, 1.3, 4.2) compared to its baseline (9.0 implants per recipient mouse, SD±0.9; $p < 0.001$). Therefore, only a proportion of the expected numbers of embryos had fully developed and initiated implantation by those times, which correlated with the fact that a small variation in embryo development was normal. Maximum expected mean implantation rates would only be possible once all embryos had become implantation-competent. Despite the lack of a zero baseline (pre-receptive period), such low levels of implantation suggested that time-points were close to the beginning of endometrial receptivity. Furthermore, the majority of blue-bands (67%) detected were graded as a 2+. Such grading was indicative of the early vascularisation and implantation.

It is worth noting that prior to 92 hours pc, the presence of implants within recipient mice was not consistently observed. More than 60% of recipient mice assessed at 88 and 90 hours pc did not result in implants. It was unclear whether this lack of implantation was a result of immature embryos, immature recipient mouse

endometrium or the mouse's incapability of implantation. However, 100% of naturally pregnant mice in Section 4.1 resulted in implants. For that reason, it was less likely that an incapability of implantation was linked to the observed lack of implants.

Within natural mouse pregnancy, it is assumed that embryo-endometrial development is synchronous, where both are initiated by the act of coitus. The approximate timing of coitus is thought to be at the mid-point of the dark-phase, which is the period of time the mouse colony is not exposed to light. However, the moment of coitus is dependent on whether the female mouse has successfully entered the estrus phase of her reproductive cycle. Therefore, it is plausible that the distinct moment of ovulation, coitus, and thus endometrial receptivity, can vary mouse-to-mouse. In context of restricted breeding, explained at the beginning of section 3.2.3, the developmental age of endometrium at the time of autopsy may be less mature than real-time pc. Such difference in endometrial maturity may account for the varied level of implantation observed at each assessed time-point. The significance of this variation is discussed further in Section 6.2.1.
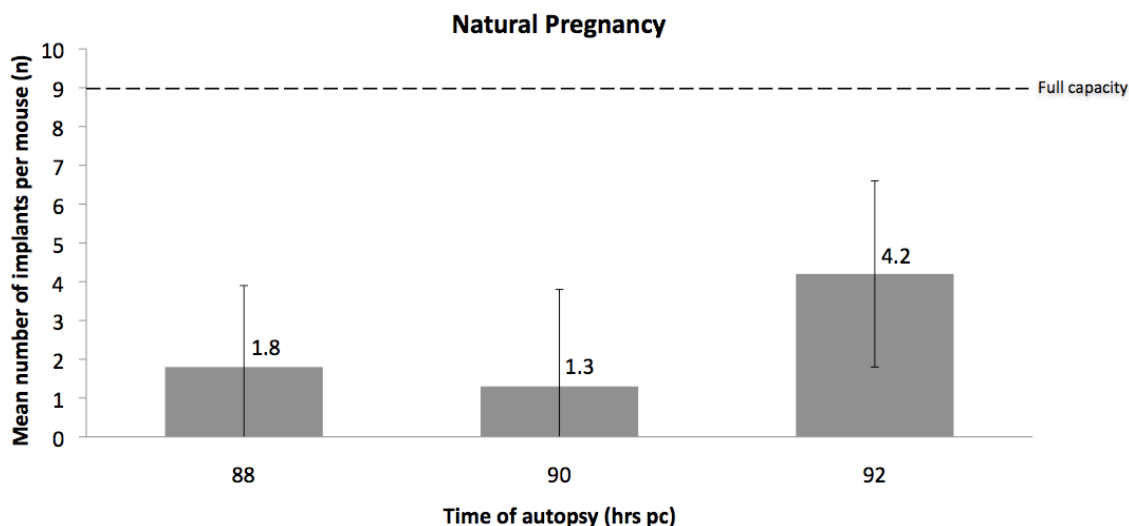
Figure 75 Mean implantation rates within natural mouse pregnancy compared to its baseline at 88, 90 and 92 hours pc. Dotted line represents baseline (day-17 pc). Error bars demonstrate one standard deviation. No significant differences were seen between time-points ($p > 0.1$). The mean numbers of implants at all time-points were significantly reduced compared to the baseline of natural pregnancy ($p \leq 0.01$).

5.2 Exploring the beginning of endometrial receptivity within synchronous and asynchronous uterine ET

The impact of altered embryo-endometrial developmental synchrony on the time of endometrial receptivity within uterine ET has not yet been published within the literature. Therefore, the purpose of the research within Section 4.2 was to determine the time endometrial receptivity began within different synchronous and asynchronous uterine ET scenarios. This included the asynchronous transfer of E3.5(-) or E4.5(-) embryos into R2.5 recipient mice; and the synchronous and asynchronous transfer of E3.5(-) or E4.5(-) embryos into R3.5 recipient mice.

The stringent design utilised to explore the beginning of endometrial receptivity provided an improved level of statistical robustness compared to the literature. As in Section 4.1, recipient mice were assessed for the presence of implants at 88, 90 or 92 hours pc. The length of time between ET and autopsy was standardised (Figure 76 and Figure 77). Embryo transfers into R2.5 recipient mice were performed at 56, 58 or 60 hours pc. Autopsies were subsequently performed 32 hours later at 88, 90 or 92 hours pc. In contrast, ETs into R3.5 recipient mice were performed at 80, 82 or 84 hours pc. Eight hours later, autopsies were performed at 88, 90 or 92 hours pc. Such experimental design was intended to ensure that within each ET scenario, donor embryos would have equal opportunity to implant prior to autopsy.

Due to the rigorous nature of experimental design, a maximum of three ETs, one designated to each assessed time-point, was possible within any one set of experiments, which from preparation to autopsy took eight days in length to complete. Thus, depending on the numbers of recipient mice required to achieve 80% statistical power (three to five mice per assessed time-point), a total of nine to fifteen recipient mice were required for each ET scenario.

A minimum of six sexually mature female mice, which were in estrus, was required on a weekly basis to generate the minimum of three pseudo-pregnant recipient mice. Given that that ovulation occurs every four to five days, 20% of available mice would be estrus at any one time. Therefore, to ensure sufficient numbers, a constant pool of approximately 40 sexually mature female mice was necessary.
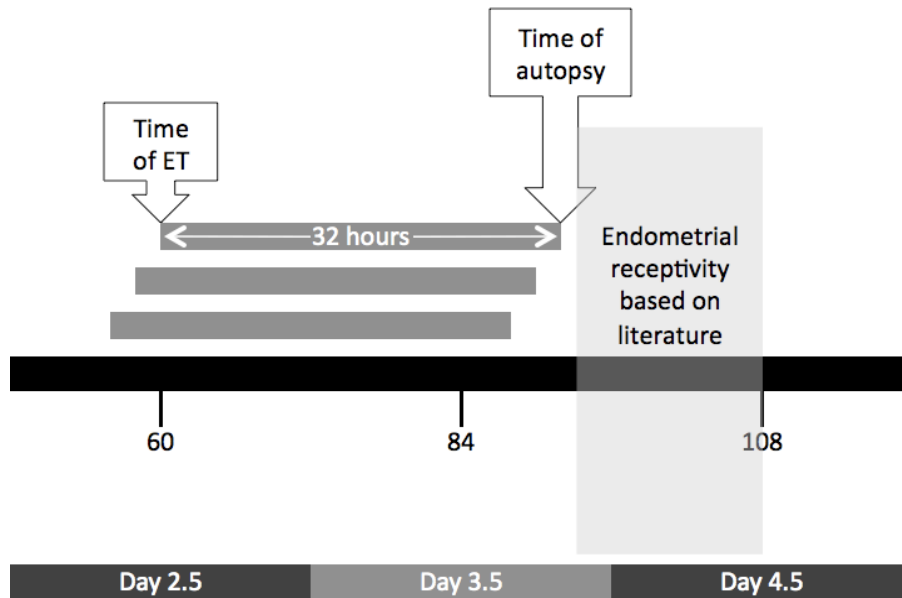
Figure 76 Experimental design identifying beginning of endometrial receptivity within R2.5 recipient mice. R2.5 recipient mice each received ten E3.5(-) or E4.5(-) embryos (five per uterine horn) at 56, 58 or 60 hours pc. Thirty-two hours later, recipient mice were assessed for the presence of implants at 88, 90 and 92 hours pc. Minimums of three recipient mice were assessed at each time-point (statistical power >80%).
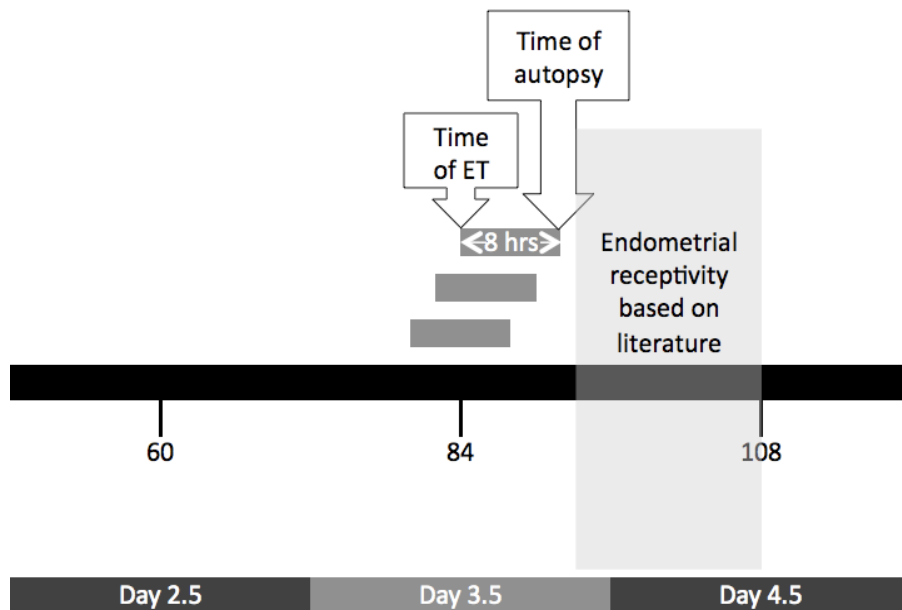
Figure 77 Experimental design identifying beginning of endometrial receptivity within R3.5 recipient mice. R3.5 recipient mice each received ten E3.5(-) or E4.5(-) embryos (five per uterine horn) at 80, 82 or 84 hours pc. Eight hours later, recipient mice were assessed for the presence of implants at 88, 90 and 92 hours pc. Minimums of three recipient mice were assessed at each time-point (statistical power >80%).

Each ET required ten normal donor embryos, which translated into at least 30 – 40 donor embryos per set of experiments. A minimum of five prepubescent female donor mice was required on a weekly basis to guarantee sufficient numbers of embryos. Therefore, a total of 90 – 150 donor embryos was necessary for each ET scenario explored, which required at least 15 – 25 donor embryo retrievals over a period of five to six weeks.

It was hoped that by implementing such a stringent experimental design the accuracy at which the beginning of endometrial receptivity was identified would be substantially greater than previously reported within the literature. Data would be compared with their corresponding baselines (day-17 pc) and would be used to reveal an insight into the mouse-to-mouse variability observed within Chapter 4.

5.2.1 Method overview:

With the assistance of an AUT Statistics Lecturer, the initial Suboptimal model trial (Section 5.2.2.4), which utilised 13 recipient mice, was used to calculate power using the PASS statistical software programme (NCSS). Sample size, and the sizes of type I (alpha) error, actual size effect and experimental error, were entered into PASS, indicating that at least three recipient mice per assessed time-point were required to achieve 80% statistical power. This translated to a minimum of three replicates per assess time-point, over four to five experimental series (weeks) per ET scenario (Figure 18). Embryos were collected from pre-pubescent donor B6CBAF1/J mice on day-1.5 pc, as per Section 3.2.7. Embryos were then *in vitro* cultured in 5% CO_2 at 37°C until required. Pseudo-pregnant B6CBAF1/J recipient mice were produced by restricted-coitus with vasectomised CBA/J male mice of proven sterility (Sections 3.2.2 and 3.2.3).

Four different synchronous and asynchronous ET scenarios were assessed (Figure 38). Ten embryos (five per uterine horn) were transferred into the uteri of pseudo-pregnant recipient mice as per Section 3.2.9. However, the time between ET and autopsy was strictly controlled. On the day of ET, R2.5 recipient mice received embryos at 56, 58 or 60 hours pc. Thirty-two hours following ET, autopsies were carried out. R3.5 recipient mice received embryos at 80, 82 or 84 hours pc. Eight hours later autopsies were performed. This meant that mice were assessed for the presence of implants at 88, 90 or 92 hours pc, as in Section 4.1. In addition to standard deviation, Chi-square tests were used to confirm any significant difference

between time-points. Fisher's exact tests were used to compare assessed time-points with the model's corresponding baseline.

5.2.2 Results

5.2.2.1 Asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice

Table 54 demonstrates that the asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice resulted in implants at 88, 90 and 92 hours pc. A mean of 8.4 implants per recipient (SD±2.6) was observed at 88 hours pc. This was followed by a mean of 9.5 implants per recipient (SD±0.5) at 90 hours pc. Finally, a mean of 8.2 implants per recipient (SD±2.5) were observed at 92 hours pc. While all three time-points were somewhat comparable to each other, Chi-square test indicated a strong tendancy towards significant differences (p = 0.075). The small reduction in mean numbers of implants observed at 88 and 92 hours pc, was significantly different to their corresponding baseling (9.4; p < 0.03). In contrast, mean numbers of implants at 90 hours pc were comparable to their baseline (p = 1.0).

In terms of individual implantation rates, 13 of the 15 recipient mice assessed resulted in an implantation rate between 8 - 10 implants per recipient mouse (Figure 78). Eight of those recipient mice resulted in the maximum possible numbers of implants (10). Despite the significant differences observed at 88 and 90 hours pc, five recipient mice resulted in maximal possible implants, where eight recipient mice resulted in 8 or more implants per mouse. Table 55 indicated embryo quality and stage of development between recipient mice and their assessed time-points were comparable. Figure 79 demonstrates that 3 of the 119 blue-bands detected were graded as a 1+ (2.5%). A further 47 (39.5%) were graded as a 2+ and the remaining 69 (58%) blue-bands were graded as a 3+.

Table 54 Mean numbers of implants following asynchronous transfer of E3.5(-) embryos (five per uterine horn) into 15 R2.5 recipient mice at 88, 90 and 92 hours pc. Mean numbers of implants were comparable between each time-point (p = 0.075). Mean numbers of implants at 88 and 92 hours pc were significantly reduced compared to their baseline (9.5; p < 0.03). Mean numbers of implants at 90 hours pc were comparable to their baseline (p = 1.0).

Time of autopsy pc (hrs)	Number of mice	Mean number of implants per recipient (±SD)	P-value compared to baseline
88	5	8.4 (±2.6)	0.025[a]
90	5	9.6 (±0.5)	1.0
92	5	8.2 (±2.5)	0.013[a]

a = significantly different p ≤ 0.05

Figure 78 Numbers and location of implants at 88, 90 and 92 hours pc following the asynchronous transfer of E3.5(-) embryos (five per uterine horn) into 15 R2.5 recipient mice. Features as per Figure 73. All, but two recipient mice achieved the baseline implantation rate.
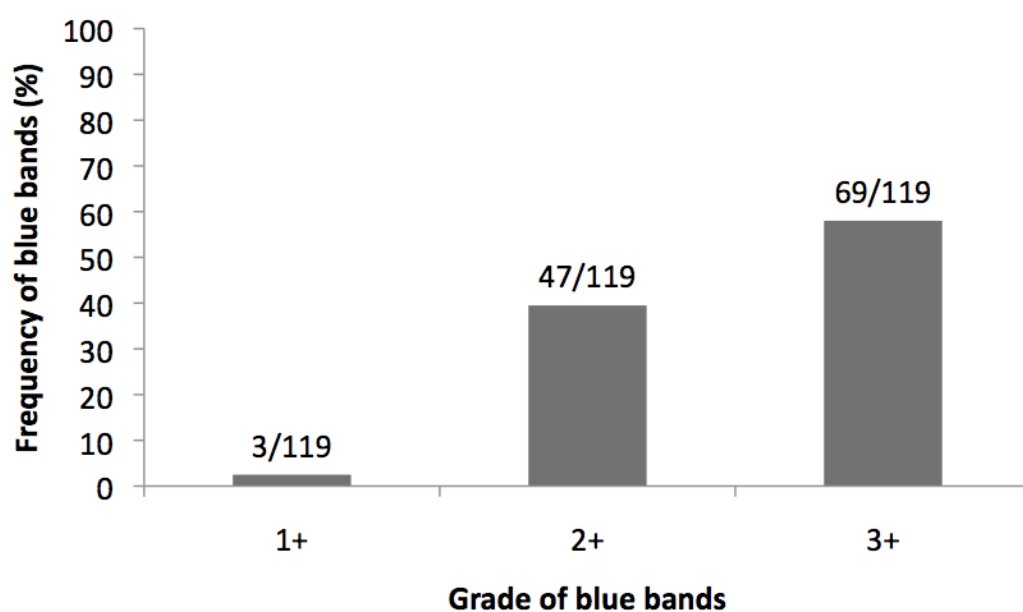
Figure 79 Total frequencies of blue-band grades on day-3.5 pc subsequent to the asynchronous transfer of E3.5(-) embryos (five per uterine horn) into 15 R2.5 recipient mice. Data included 88, 90 and 92 hours pc (day-3.5 pc). Values above bars are the numbers of bands over the total numbers of bands detected. Grades as per Protocol 9.7. Fifty-eight percent of blue-bands were graded as a 3+.

Table 55 Grade allocation of E3.5(-) embryos transferred into R2.5 recipient mice for assessment at 88, 90 and 92 hours pc. Data grouped by assessment time and lowest to highest numbers of implants per recipient mouse. Each recipient mouse represented one replicate for each time-point over six experimental series (Figure 18). Donor embryos were retrieved from a pool of prepubescent donor mice for each experimental series. Embryo grades, indicating stage of development and quality, were allocated as per Section 3.2.8 (Table 17). Key: Recip, recipient; Exp, experimental; M, morula; 5A – 3A, early blastocyst.

Time of autopsy pc (hrs)	Recip order	Exp series	Numbers per recipient mouse				Implants
			Embryo grade at time of ET (%)				
			M[a]	5A	4A	3A	
92	10	4	7 (70)	-	2 (20)	1 (10)	4
92	1	1	3 (30)	-	7 (70)	-	8
92	7	3	6 (60)	-	3 (30)	1 (10)[b]	9
92	13	5	7 (70)	-	3 (30)[d]	-	10
92	15	6	5 (50)	-	5 (50)	-	10
90	9	4	3 (30)	-	5 (50)	2 (20)[c]	9
90	12	5	8 (80)	-	2 (20)	-	9
90	14	6	10 (100)	-	-	-	10
90	4	2	3 (30)	-	7 (70)	-	10
90	6	3	8 (80)	-	-	2 (20)[c]	10
88	2	1	9 (90)	-	1 (10)	-	4
88	5	3	8 (80)	-	1 (10)[b]	-	8
88	3	2	7 (70)	-	3 (30)	-	10
88	8	4	6 (60)	-	2 (20)	2 (20)	10
88	11	5	8 (80)	-	2 (20)	-	10

a = all morula A-grade
b = prematurely hatching, but cell integrity in tack.
c = one prematurely hatching, but cell integrity in tack.
d = one B-grade

5.2.2.2 Asynchronous transfer of E4.5(-) embryos into R2.5 recipient mice

The asynchronous transfer of E4.5(-) embryos into R2.5 recipient mice also resulted in the detection of implants at 88, 90 and 92 hours pc (Table 56). A mean number of 7.8 implants per recipient (SD±2.1) were detected at 88 hours pc. Assesed time-point, 90 hours pc, resulted in a mean of 9.7 implants per recipient (SD±0.6). At 92 hours pc, a mean of 8.5 implants per recipient mouse was detected (SD±1.9). The small differences observed between each time-point had a strong tendancy towards significance (p = 0.076). Time-points 88 and 92 hours pc resulted in a small, but significant decrease compared to their corresponding baseline (9.8; p < 0.02). Mean numbers of implants at 90 hours pc were comparable to their baseline (p = 1.0).

Of the 11 recipient mice assessed, 75% (8/11) resulted in 8 or more implants per recipient mouse (Figure 79). Five of those recipient mice resulted in the maximum possible numbers of implants (10), which represented 45% to the total population of recipient mice. Table 57 indicated embryo quality and stage of development between recipient mice and their assessed time-points were comparable. As indicated in Figure 81, 3 (4%) of the 84 bands detected were graded as a 1+. Sixteen (19%) were graded as a 2+ and the remainder of blue-bands were given a 3+ grade (65/84, 77%).

Figure 80 Numbers and location of implants at 88, 90 and 92 hours pc following the asynchronous transfer of E4.5(-) embryos (five per uterine horn) into 11 R2.5 recipient mice. Features as per Figure 73. Seven of the 11 recipient mice were comparable to the baseline (9.8).

Table 56 Mean numbers of implants following asynchronous transfer of E4.5(-) embryos (five per uterine horn) into 11 R2.5 recipient mice at 88, 90 and 92 hours pc. Differences observed between time-point had a strong tendancy towards significance (p = 0.076). Time-points, 88 and 92 hours pc, were significantly reduced compared to their baseline (9.8; p < 0.02). Time-point, 90 hours pc, was comparable to the corresponding baseline (p = 1.0).

Time of autopsy pc (hrs)	Number of mice	Mean number of implants per recipient (±SD)	P-value compared to baseline
88	4	7.8 (±2.1)	< 0.001[a]
90	3	9.7 (±0.6)	1.0
92	4	8.5 (±1.9)	0.016[a]

a = significantly different p ≤ 0.05

Figure 81 Total frequencies of blue-band grades on day-3.5 pc subsequent to the asynchronous transfer of E4.5(-) embryos (five per uterine horn) into 11 R2.5 recipient mice. Data includes 88, 90 and 92 hours pc (day-3.5 pc). Features as per Figure 79. Seventy-seven percent of blue-bands were graded as a 3+.

Table 57 Grade allocation of E4.5(-) embryos transferred into R2.5 recipient mice for assessment at 88, 90 and 92 hours pc. Data grouped by assessment time and lowest to highest numbers of implants per recipient mouse. Each recipient mouse represented one replicate for each time-point over six experimental series (Figure 18). Donor embryos were retrieved from a pool of prepubescent donor mice for each experimental series. Embryo grades, indicating stage of development and quality, were allocated as per Section 3.2.8 (Table 17). Key: Recip, recipient; Exp, experimental; 2A – 1A late blastocyst; HgB, hatching blastocyst; HdB, hatched blastocyst.

Time of autopsy pc (hrs)	Recip order	Exp series	Numbers per recipient mouse				Implants
			Embryo grade at time of ET (%)				
			2A	1A	HgB	HdB	
92	7	4	-	-	8 (80)	2 (20)	6
92	6	4	-	-	8 (80)	2 (20)	8
92	10	5	-	-	8 (80)	2 (20)	10
92	11	6	-	-	8 (80)	2 (20)	10
90	5	4	-	-	8 (80)	2 (20)	9
90	9	5	-	-	8 (80)	2 (20)	10
90	1	1	-	-	10 (100)	-	10
88	8	5	-	-	8 (80)	2 (20)	6
88	3	3	-	-	10 (100)	-	6
88	4	4	-	1 (10)	9 (90)	-	9
88	2	2	1 (10)	2 (20)	7 (70)	-	10

5.2.2.3 Synchronous transfer of E3.5(-) embryos into R3.5 recipient mice

Implants were detected at 88, 90 and 92 hours pc following the synchronous transfer of E3.5(-) embryos into R3.5 recipient mice (Table 58). A mean of 1.6 implants per recipient (SD±1.1) were detected at 88 hours pc, followed by 0.8 implants per recipient (SD±1.3) at 90 hours pc. A mean of 0.8 implants per recipient (SD±1.0) was also detected at 92 hours pc. All time-points were comparable to each other ($p = 0.32$). Mean numbers of implants at 88 hours pc were comparable to the corresponding baseline (2.2; $p = 0.509$). In contrast, time-points 90 and 92 hours pc were significantly reduced compared to their baseline ($p < 0.04$).

Fifty-seven percent of recipient mice (8/14) resulted in implants at 88, 90 and 92 hours pc (Figure 82). No implants were observed in the remainder of recipient mice. Table 59 indicated embryo quality and stage of development between recipient mice and their assessed time-points were comparable. As seen in Figure 83, 40% of blue-bands were graded as a 1+ (6/15). The majority of blue-bands were graded as a 2+ (53%, 8/15) and the sole remaining blue-band was graded as a 3+ (7%, 1/15).
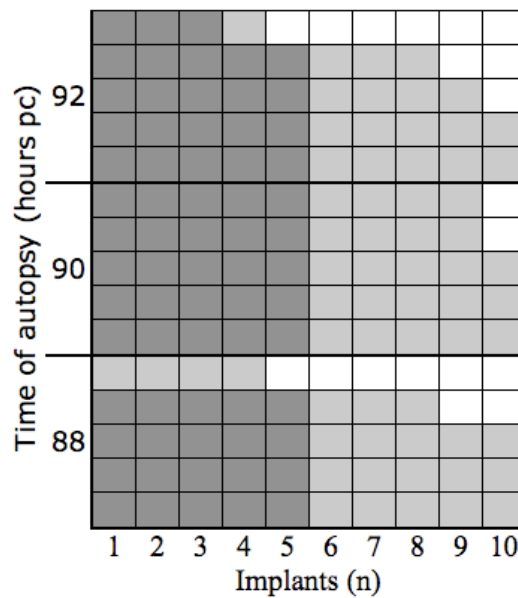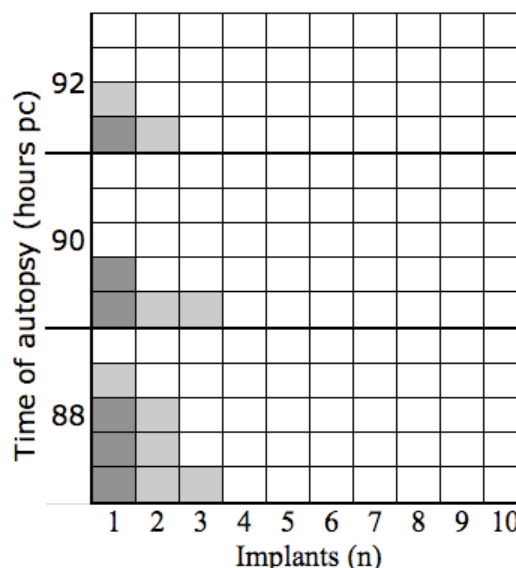

Figure 82 Numbers and location of implants at 88, 90 and 92 hours pc following the synchronous transfer of E3.5(-) embryos (five per uterine horn) into 14 R3.5 recipient mice. Features as per Figure 73. Forty-three percent of recipient mice resulted in zero implants.

Table 58 Mean numbers of implants at 88, 90 and 92 hours pc following synchronous transfer of E3.5(-) embryos (five per uterine horn) into 14 R3.5 recipient mice. Time-points were comparable (p = 0.32). Time-point, 88 hours pc, was comparable to the corresponding baseline (2.2; p = 0.509). All other time-points were significantly reduced compared to baseline data (p < 0.04).

| Time of autopsy pc (hrs)	Number of mice	Mean number of implants per recipient (±SD)	P-value compared to baseline
88	5	1.6 (±1.1)	0.509
90	5	0.8 (±1.3)	0.036[a]
92	4	0.8 (±1.0)	0.016[a]

a = significantly different p ≤ 0.05
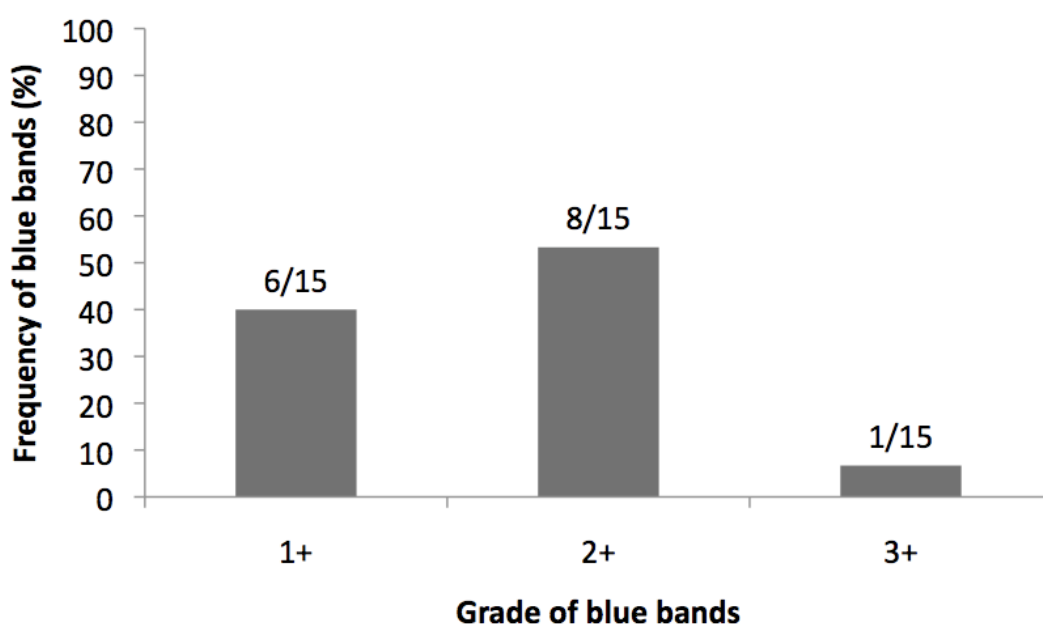
Figure 83 Total frequencies of blue-band grades on day-3.5 pc subsequent to the synchronous transfer of E3.5(-) embryos (five per uterine horn) into 14 R3.5 recipient mice. Data included 88, 90 and 92 hours pc (day-3.5 pc). Features as per Figure 79. Fifty-three percent of blue-bands were graded as a 2+.

Table 59 Grade allocation of E3.5(-) embryos transferred into R3.5 recipient mice for assessment at 88, 90 and 92 hours pc. Data grouped by assessment time and lowest to highest numbers of implants per recipient mouse. Each recipient mouse represented one replicate for each time-point over six experimental series (Figure 18). Donor embryos were retrieved from a pool of prepubescent donor mice for each experimental series. Embryo grades, indicating stage of development and quality, were allocated as per Section 3.2.9 (Table 17). Key: Recip, recipient; Exp, experimental; M, morula; 5A – 3A, early blastocyst.

Time of autopsy pc (hrs)	Recip order	Exp series	Numbers per recipient mouse				Implants
			Embryo grade at time of ET (%)				
			M[a]	5A	4A	3A	
92	10	4	5 (50)	-	3 (30)	2 (20)	0
92	13	5	5 (50)	-	4 (40)	3 (30)	0
92	5	2	5 (50)	-	3 (30)	2 (20)[b]	1
92	2	1	8 (80)	-	1 (10)	1 (10)	2
90	4	2	8 (80)	-	1 (10)	1 (10)	0
90	12	5	7 (70)	-	2 (20)[c]	1 (10)	0
90	14	6	5 (50)	-	4 (40)	1 (10)	0
90	9	4	7 (70)	-	1 (10)	2 (20)	1
90	7	3	8 (80)	-	2 (20)	-	3
88	6	3	8 (80)	-	2 (20)	-	0
88	8	4	7 (70)	-	2 (20)	1 (10)	1
88	1	1	10 (100)	-	-	-	2
88	3	2	10 (100)	-	-	-	2
88	11	5	8 (80)	-	2 (20)	-	3

a = all morula A-grade
b = one prematurely hatching, but cell integrity in tack.
d = one B-grade

5.2.2.4 Asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice

Following the asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice, implants were detected at 88, 90 and 92 hours pc. A small increase in the mean number of implants per recipient was seen at each time-point (Table 60). At 88 hours pc, a mean of 0.4 implants per recipient (SD±0.9) was seen. A small increase was seen at 90 hours pc where a mean of 1.3 implants per recipient (SD±1.5) was observed. The mean number of implants per recipient at 92 hours pc also increased (3.3, SD±1.7). Chi-square tests indicated these differences were significant ($p < 0.001$). In addition, the mean numbers of implants at 88, 90 and 92 hours pc were significantly reduced compared to the corresponding baseline (6.2, Table 32; $p \leq 0.002$).

In context of individual data, implants were observed in 54% (7/13) of recipient mice (Figure 84). Numbers of implants per recipient mouse ranged from 1 – 5 implants, although most recipient mice resulted in 3 or less implants. Table 61 indicated embryo quality and stage of development between recipient mice and their assessed time-points were comparable. Figure 85 demonstrates that the majority of blue-bands were graded as a 2+ (60%, 12/20). The remaining blue-bands were graded as either a 1+ (30%, 6/20) or a 3+ (10%, 2/20).
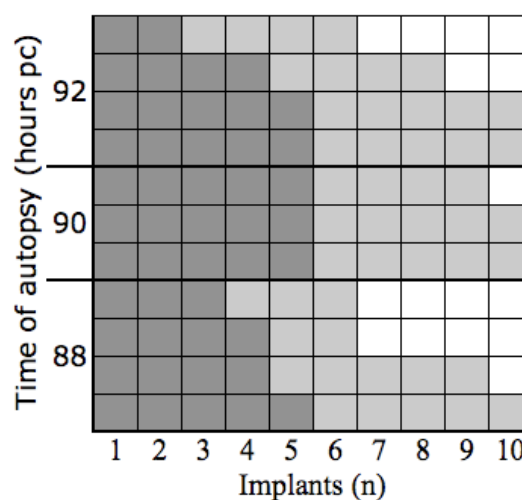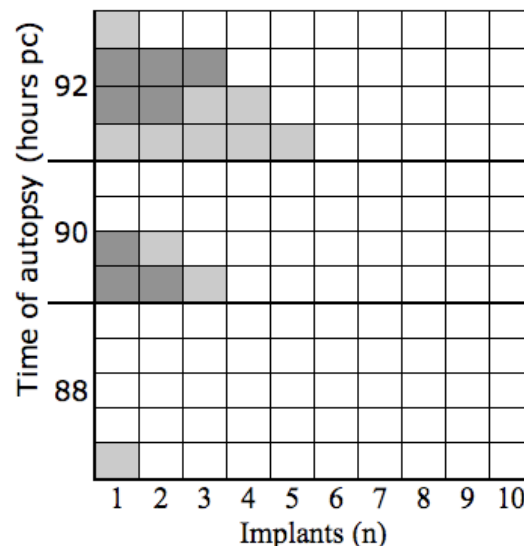

Figure 84 Numbers and location of implants at 88, 90 and 92 hours pc following the asynchronous transfer of E4.5(-) embryos (five per uterine horn) into 13 R3.5 recipient mice. Features as per Figure 73. Numbers of recipient mice demonstrating implantation increased over time.

Table 60 Mean numbers of implants at 88, 90 and 92 hours pc following asynchronous transfer of E4.5(-) embryos (five per uterine horn) into 13 R3.5 recipient mice. Differences observed between time-points were significant (p < 0.001). All time-points significantly reduced compared to the corresponding baseline (6.2; p ≤ 0.002).

Time of autopsy pc (hrs)	Number of mice	Mean number of implants per recipient (±SD)	P-value compared to baseline
88	5	0.4 (±0.9)	< 0.001[b]
90	4	1.3 (±1.5)	< 0.001[b]
92	4	3.3 (±1.7)	0.002[a]

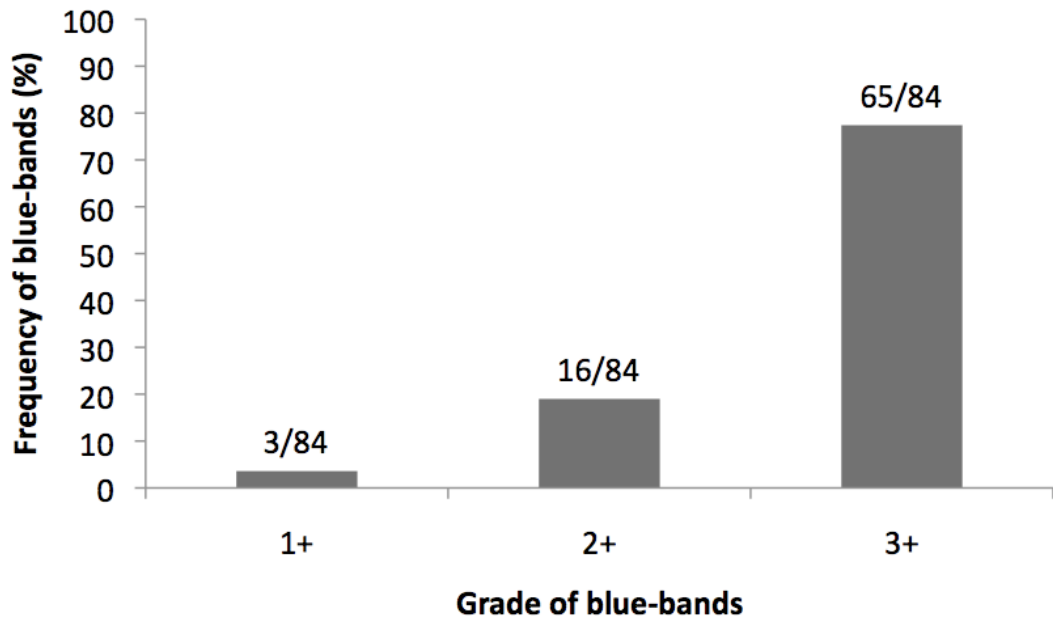a = values significantly difference from baseline p ≤ 0.002
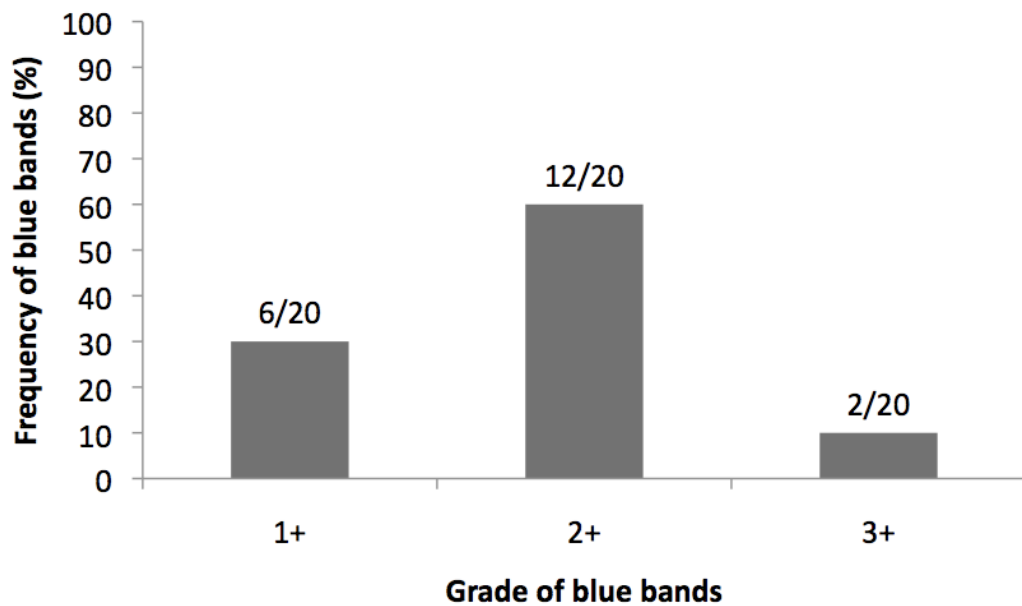
Figure 85 Total frequencies of blue-band grades on day-3.5 pc subsequent to the asynchronous transfer of E4.5(-) embryos (five per uterine horn) into 13 R3.5 recipient mice. Data included 88, 90 and 92 hours pc (day-3.5 pc). Features as per Figure 79. Sixty percent of blue-bands were graded as a 2+.

Table 61 Grade allocation of E4.5(-) embryos transferred into R3.5 recipient mice for assessment at 88, 90 and 92 hours pc. Data grouped by assessment time and lowest to highest numbers of implants per recipient mouse. Each recipient mouse represented one replicate for each time-point over six experimental series (Figure 18). Donor embryos were retrieved from a pool of prepubescent donor mice for each experimental series. Embryo grades, indicating stage of development and quality, were allocated as per Section 3.2.8 (Table 17). Key: Recip, recipient; Exp, experimental; 2A – 1A late blastocyst; HgB, hatching blastocyst; HdB, hatched blastocyst.

Time of autopsy pc (hrs)	Recip order	Exp series	Numbers per recipient mouse				
			Embryo grade at time of ET (%)				Implants
			2A	1A	HgB	HdB	
92	8	4	-	-	10 (100)	-	1
92	14	7	1 (10)	1 (10)	8 (80)	-	3
92	12	6	-	-	10 (100)	-	4
92	11	5	-	-	10 (100)	-	5
90	5	3	-	3 (30)	7 (70)	-	0
90	7	4	-	-	10 (100)	-	0
90	3	2	-	1 (10)	9 (90)	-	2
90	10	5	-	-	10 (100)	-	3
88	2	2	-	2 (20)	8 (80)	-	0
88	4	3	-	-	10 (100)	-	0
88	6	4	-	1 (10)	8 (80)	1 (10)	0
88	9	5	-	1 (10)	9 (90)	-	0
88	1	1	-	1 (10)	9 (90)	-	1

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5.2.3 Interpretation for beginning of endometrial receptivity within synchronous and asynchronous ET

Within each ET scenario, data suggested that the endometrium could be receptive by 88 hours pc. Despite the small but significant differences indicated by Chi-square tests, three of the four scenarios resulted in mean numbers of implants similar to their corresponding baselines. Data suggested each time-point fell further along the period of endometrial receptivity rather than at the beginning. Furthermore, grades of blue-bands varied between each ET scenario, which indicated that the extent of vascularisation and implantation was varied. Thus, data suggested different synchronous and asynchronous ET scenarios impacted the timing of endometrial receptivity in pseudo-pregnant recipient mice. The following paragraphs will explain data in greater depth.

Subsequent to the asynchronous transfer of E3.5(-) or E4.5(-) embryos into R2.5 recipient mice, implants were detected from 88 hours pc (Figure 86). Although some significant differences were observed, each time-point resulted in mean numbers of implants similar to their corresponding baselines (9.4, SD±1.1; 9.8, SD±0.5). Following the transfer of E3.5(-) embryos into R2.5 recipient mice, 13/15 (87%) recipient mice resulted in 8 or more implants per mouse. Following the transfer of their E4.5(-) counterparts, 8/11 (73%) recipient mice also resulted in 8 or more implants per mouse. This indicated that most recipient mice resulted in maximum expected mean numbers of implants by 88 hours pc. Data, therefore, suggested that each time-point was inclusive of the receptive period, and were located further along the period of endometrial receptivity.

The advancement of endometrial receptivity was confirmed further by grading of the blue-bands. Following the transfer of E3.5(-) embryos, more than 58% of blue-bands were graded as a 3+. Likewise, 75% of blue-bands following the transfer of E4.5(-) embryos were graded as a 3+. Not only were blue-bands clearer than those found in natural mouse pregnancy (≤ 2+, Figure 74), but they also covered a greater proportion of the uterine horn circumference. Consequently, data suggested implants were more advanced than those found in natural mouse pregnancy and that endometrial receptivity had begun prior to 88 hours pc.

Graph A - E3.5(-) embryos transferred into R2.5 recipient mice
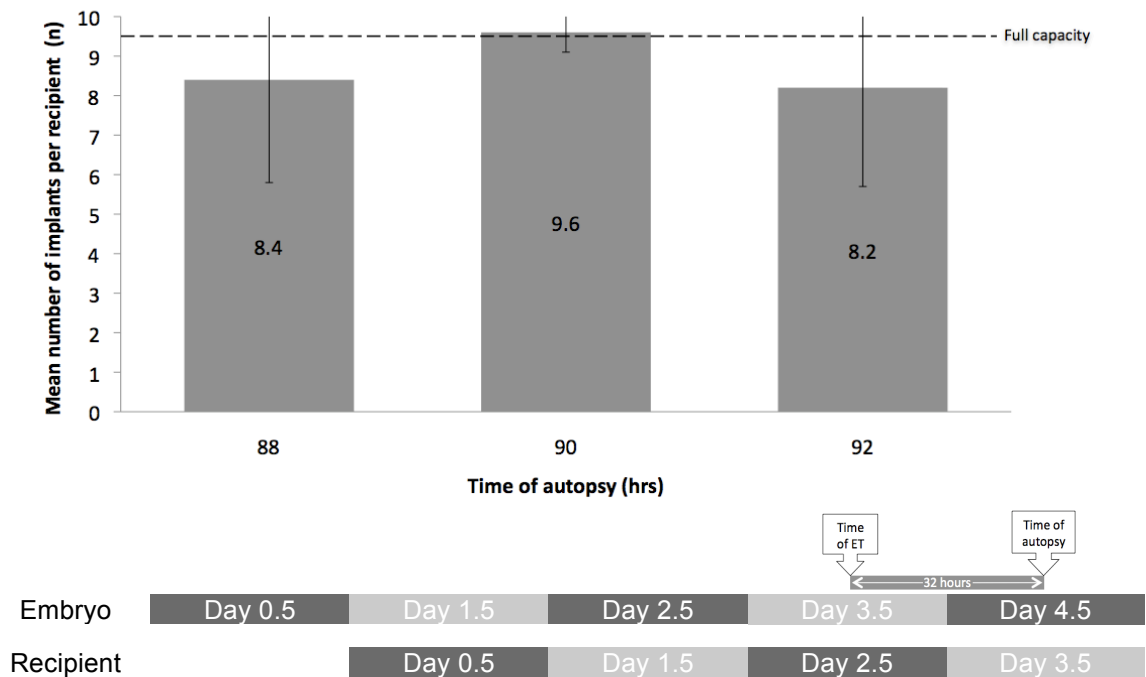
Graph B - E4.5(-) embryos transferred into R2.5 recipient mice
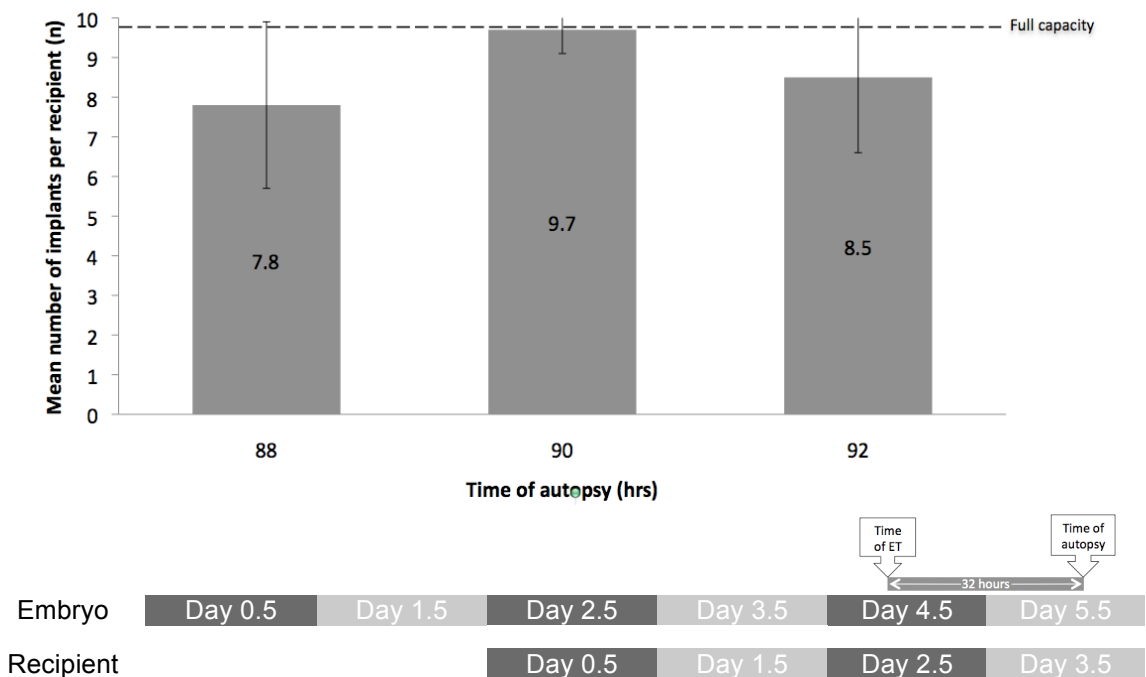
Figure 86 Mean implantation rates at 88, 90 and 92 hours pc subsequent to the asynchronous transfer of E3.5(-) or E4.5(-) embryos into R2.5 recipient mice compared to its baseline. Dotted line is the expected baseline of natural pregnancy (day-17 pc). Error bars demonstrate one standard deviation. Bottom section of figure indicates experimental design.

It was difficult to ascertain whether the asynchronous transfers of E3.5(-) or E4.5(-) embryos into R2.5 recipient mice shared a similar variation in endometrial maturity at the time of autopsy, as was demonstrated within natural mouse pregnancy (Section 4.1). The high implantation rates observed in either ET scenario contributed to this lack of clarity. Seventy-seven percent of R2.5 recipient mice resulted in implantation rates greater than 8 implants per recipient mouse. Furthermore, maximum possible numbers of implants were observed in 50% of recipient mice (10 implants per recipient mouse).

The synchronous transfer of E3.5(-) embryos into R3.5 recipient mice also resulted in implants from 88 hours pc onwards (Figure 87). Thus, data suggested each time-point was inclusive of the receptive period. Additionally, the mean numbers of implants were also comparable to the corresponding baseline (2.2, SD±2.3). However, 43% of recipient mice assessed lacked implants. Eighty-three percent of those recipient mice exhibited zero implantation at 90 and 92 hours pc. Yet in contrast, 40% of recipient mice that were utilised to determine the corresponding baseline (day-17 pc) also demonstrated a lack of implants. Therefore, the lack of implantation in a proportion of recipient mice was not considered abnormal for the synchronous transfer of E3.5(-) into R3.5 recipient mice. It is worth noting, however, that increasing the number of replicates (recipient mice) for each assessed time-point could have improved clarity. Nonetheless, the number of implants per recipient mouse could not reliably suggest where time-points fell within the receptive period, nor could such numbers elucidate any variation in the maturity of endometrial development at the time of autopsy.

E3.5(-) embryos transferred into R3.5 recipient mice
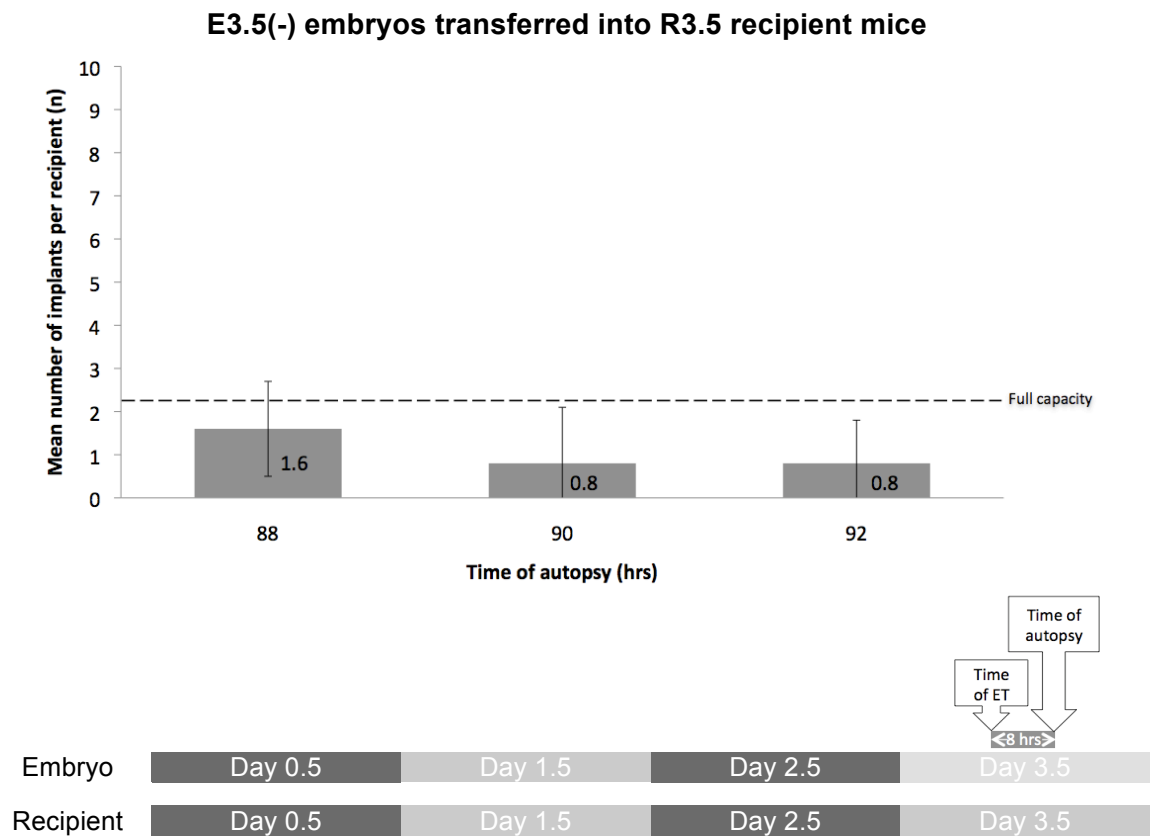
Figure 87 Mean numbers of implants at 88, 90 and 92 hours pc following synchronous transfer of E3.5(-) embryos into R3.5 recipient mice compared to its baseline. Features as per Figure 86.

Despite the limitations of the data, the majority of blue-bands (93%) received a grade no higher than a 2+. This level of band intensity was comparable to natural mouse pregnancy, where grades were no more than a 2+. Therefore, data suggested that implantation advancement was comparable to natural mouse pregnancy. For that reason, it is most likely that each time-point was located towards the beginning of endometrial receptivity.

The presence of implants was once more detected from 88 hours pc subsequent to the asynchronous transfer of E4.5(-) embryos into R3.5 mice (Figure 88). Unlike the first three ET scenarios, mean implantation rates were reduced compared to their corresponding baseline (6.2, SD±2.6; Figure 88). However, these values increased over time, which reflected a rise in the numbers of embryos that were able become implantation-competent and initiate implantation over time. Thus, not only did data indicate that endometrial receptivity had begun by 88 hours pc, it also suggested that each time-point was located early-on in endometrial receptivity. The grading of blue-bands further corroborated this finding. Ninety percent of blue-bands were graded no more than a 2+, which was comparable to natural mouse pregnancy. This suggested that implantation advancement was similar to that of natural mouse pregnancy.

While mean data indicated that endometrial receptivity could begin by 88 hours pc, numerous individual recipient mice did not demonstrate implantation. Furthermore, individual implantation rates varied substantially mouse-to-mouse. A lack of implantation did not reflect the corresponding baseline data (day-17 pc), where implantation was observed within 100% of recipient mice. At 88 hours pc, only 20% of recipient mice assessed exhibited implantation. Two hours later (90 hours pc), the percentage of recipient mice that demonstrated implants had increased to 50%. Finally, at 92 hours pc, implants were detected in 100% of recipient mice. Consequently, data suggested that the endometrium was consistently receptive by 92 hours pc, but could also be receptive by 88 hours pc. As previously discussed in Section 5.1, coitus was restricted to ten to eight hours following the mid-point of the dark phase, the time of first male-female contact. If coitus has occurred later on in the dark phase, it was possible that recipient endometrium had not yet become receptive by the time of autopsy, resulting in zero implantation. However, similar to the inconsistent implantation rates observed on day-17 pc (baselines), individual implantation rates at 92 hours pc ranged from 1 – 5 implants per recipient mouse.

Therefore, it was important to consider all possible reasons for such inconsistent levels of implantation. For that reason, Section 6.2 will critically analyse the potential factors contributing towards implantation rate inconsistencies, discuss their significance and suggest what they could reveal of day-17 pc data.
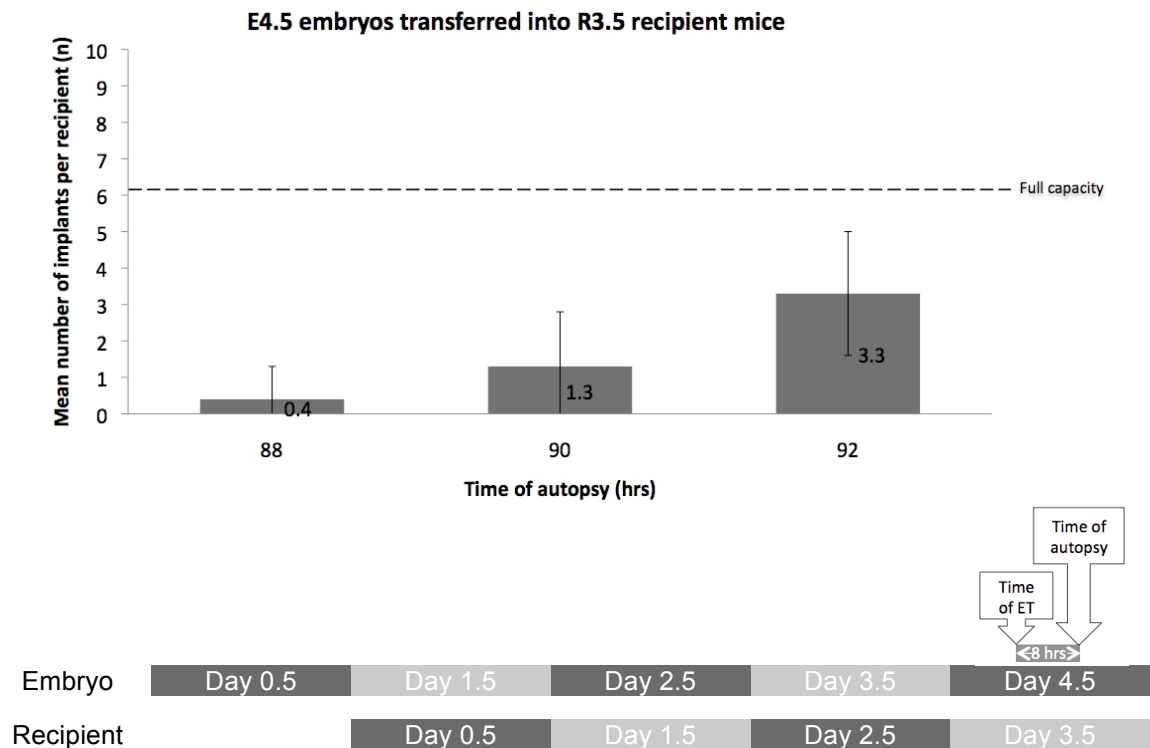
Figure 88 Mean numbers of implants at 88, 90 and 92 hours pc following asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice compared to its baseline. Features as per Figure 86. Mean number of implants at 88 hours pc was significantly reduced compared to at 92 hours pc (p = 0.039). Mean numbers of implants at all time-points were significantly reduced compared to the baseline (p ≤ 0.040).

6   Discussion

6.1 Optimal and suboptimal in vivo implantation murine models

For a model, suitable to explore IVF-intervention potential, to be considered as standard, the model needed to demonstrate both consistent implantation rates and the ability to detect consequences of the IVF-intervention of interest. Fundamental knowledge surrounding murine implantation rates is also essential - for you cannot effectively manipulate or control what you do not understand. In addition, the numerous variables between research groups highlighted a need to establish this understanding experimentally rather than relying on the literature alone. For this reason, Chapter 4 compared the outcomes on day-17 pc of natural mouse pregnancy with a selection of optimal and suboptimal *in vivo* implantation murine models, whose embryo-endometrial developmental synchrony had been altered. These models included: 1) ET into SO recipient mice (advanced endometrium); 2) progesterone antagonist administration following ET (delayed endometrium); and 3) synchronous and asynchronous ET (shift of donor embryo and recipient endometrial developmental age at the time of transfer). Findings led to the selection of the most suitable optimal and suboptimal models, which were assessed for their ability to detect negative or positive consequences of manipulated embryos within Section 4.5 and 4.6. The following discussion collates the findings of Chapter 4, revealing greater insight into murine implantation rates, the inconsistencies of existing models and the overall ability held by the Optimal and Suboptimal Model to explore the IVF-intervention potential of embryo manipulation. Thus, findings helped establish where holes lay within current knowledge and subsequent research direction, which included the beginning of endometrial receptivity (Chapter 5).

6.1.1   Mean murine implantation rates are dependent upon both the time required and available for embryo implantation-competency

The findings of this research highlight the importance of concurrently considering both the time required, and available, for embryo implantation-competency when targeting certain levels of implantation within *in vivo* implantation murine models. In contrast to the literature, mean implantation rates within models were clearly influenced by both the time required and available for embryo implantation-competency and implantation.

In the case of models that were suitable to explore the potential of embryo manipulation, certain levels of implantation were more appropriate than others to explore different aspects of that IVF-intervention's potential. For example, those models that exhibited optimal mean implantation rates could be appropriate to explore the safety of embryo manipulation. In contrast, those models that demonstrated suboptimal mean implantation rates could be suitable to assess the potential embryo manipulation held in restoring optimal levels of implantation. The following discussion will draw attention towards the need to consider both time required and available for embryo implantation-competency when targeting a certain level of implantation. These findings contribute towards the foundational knowledge surrounding murine implantation, which is necessary to establish standardised *in vivo* implantation murine models.

The literature has long recognised that implantation is dependent on the perfect synchrony of embryo-endometrial development (Armant, 2005; Psychoyos, 1973a, 1973b, 1986; Yoshinaga, 1988). Within natural mouse pregnancy, this developmental synchrony is reliant on hormone responses and feedback loops associated with the hypothalamic-anterior pituitary-gonadal axis (Hawkins & Matzuk, 2008; Wang & Dey, 2006). Although this axis plays a role in successful implantation within ET, embryo-endometrial developmental synchrony is primarily dependent on the developmental age of donor embryos and recipient endometrium at the time of transfer (McLaren & Michie, 1955; Wakuda et al., 1999). The extent of this developmental synchrony translates to how much time is required and available for donor embryos to become implantation-competent and implant. As illustrated in Figure 89, when the time required exceeds the time available to reach embryo implantation-competency, implantation is not possible. In contrast, if the time required is less or comparable to that available, implantation is possible. However, in the case where implantation is possible, how the time required and that available for embryo-implantation competency, combines to influence the level of implantation is somewhat unclear within the literature (Goto, et al., 1993; Lambers, et al., 2007; Liu, et al., 2003; Liu, et al., 2008; Matorras, et al., 2005; Song, et al., 2007; Wakuda, et al., 1999; Ye, 2006). Therefore, it was important to clarify the combined influence of the time required and available for embryo implantation-competency on mean implantation rates within this research.
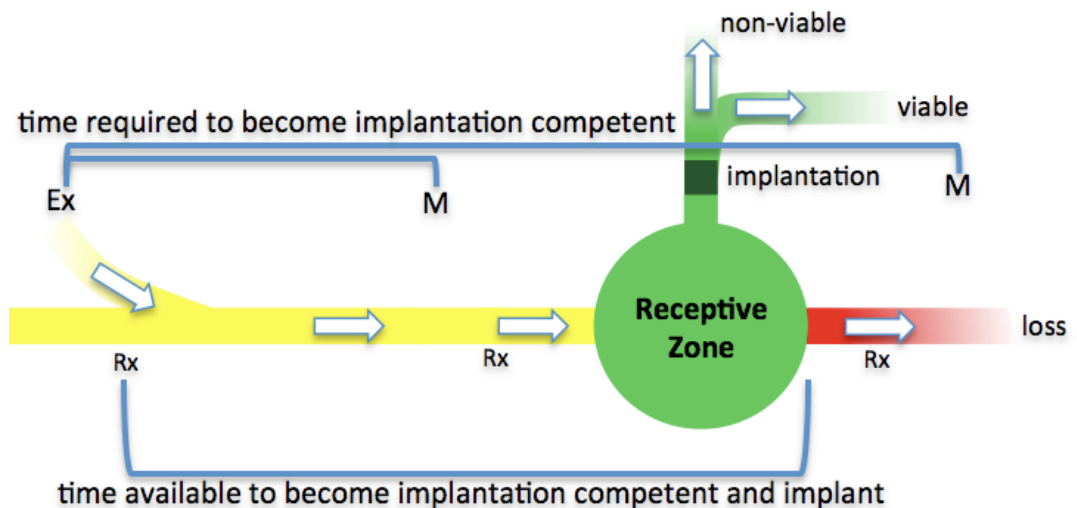
Figure 89 Schematic illustrating the impact of time required and available for embryo implantation-competency on implantation opportunity. If the time required for embryo implantation-competency exceeds the time available, implantation does not occur. If the time required is less than or comparable to what is available, implantation can occur. Key: E = embryo; R = recipient endometrium; x = developmental age days pc; M = implantation-competent embryo.

The mean implantation rates exhibited by synchronous and asynchronous ET, and ET into SO-recipient mice confirmed the need to consider both the time required and available for embryo implantation-competency when targeting a certain level of implantation (Table 62). Similar to the observations of Goto et al. (1993) and McLaren and Michie (1955), reduced implantation rates correlated with an increase in the time required by donor embryos to reach implantation-competency and implant. For example: following the asynchronous and synchronous transfer of E4.5(-) or E3.5(-) embryos into R3.5 recipient mice, 6.2 and 2.2 mean numbers of implants per recipient mouse resulted respectively. While the time available to both groups of embryos was comparable, the time required to reach implantation-competency differed by up to 24 hours. As a consequence, a proportion of E3.5(-) embryos were unable to become implantation-competent and implant prior to the end of endometrial receptivity, resulting in the reduced level of implantation. In contrast, E4.5(-) embryos had plenty of time to become implantation-competent by the beginning of endometrial receptivity. For this reason, E4.5(-) embryos had a greater opportunity to implant compared to E3.5(-) embryos, and would result in increased mean implantation rates.

In contrast to the synchronous transfer of E3.5(-) into R3.5 recipient mice, mean implantation rates increased dramatically when a comparable group of embryos were transferred into R2.5 recipient mice (9.4 implants per recipient mouse; Table 62). Although the time required by both groups of embryos was comparable between

both ET scenarios, the time available within R2.5 recipient mice was up to 24 hours longer than R3.5 recipient mice. Thus a greater proportion of E3.5(-) were able to become implantation-competent and implant prior to the end of endometrial receptivity. For that reason, research indicated a correlation between increased implantation rates as a consequence of an increased length of time available for embryo implantation-competency, and thus greater implantation opportunity.

Table 62 Impact of altered embryo-endometrial developmental synchrony on implantation rates as a consequence of synchronous and asynchronous ET. Implantation rates were expressed as mean numbers of implants per recipient mouse (±SD).

		Embryo developmental age	
		E3.5(-)	E4.5(-)
Recipient developmental age	R2.5	9.4 (±1.1)	9.8 (±0.5)
	R3.5	2.2 (±2.3)*	6.2 (±2.6)*

*Significantly less than natural mouse pregnancy, $p \leq 0.014$

It was important to note that the improved level of implantation subsequent to the asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice, was not simply a product of asynchronous embryo-endometrial development. For example, the transfer of E3.5(-) embryos into R2.5 recipient mice shared the same extent of embryo-endometrial developmental asynchrony as the transfer of E4.5(-) embryos into R3.5 recipient mice. However, their mean implantation rates differed substantially (9.4 verse 6.2 implants per recipient mouse). Given that E4.5(-) embryos would only require a minimal length of time to become implantation-competent following ET, it was most likely that the extra 24 hours available for that implantation-competency within R2.5 recipient mice held the greatest influence on level of implantation.

Embryo transfer into SO-recipient mice was also able to demonstrate a link between the time available for implantation-competency and implantation rates. Following ET into SO-recipient mice (3.3 implants per recipient mouse) compared to unmanipulated recipient mice (9.4 implants per recipient mouse; Table 63). Superovulation of female mice is thought to accelerate endometrial development, resulting in an advanced endometrium at the time of ET (Bourgain & Devroey, 2003; Kelley, et al., 2006; KNg,

et al., 2000; Oehninger, 2008).   Therefore, less time would have been available for embryo implantation-competency and implantation compared to non-superovulated female mice.   Thus reflecting the reduced implantation rates observed within this research.

Table 63 Impact of altered embryo-endometrial developmental synchrony on implantation rates as a consequence of advanced or delayed endometrium. Superovulation (SO) of sexually mature recipient mice, with 10IU of PSMG and 10IU of hCG, can generate an advanced endometrium. Progesterone antagonist administration (PA), at 2.0×10^{-5} g / 10 g of body weight one-day post-transfer, can generate a delayed endometrium.

E3.5(-) embryos

R2.5 recipient mice		E3.5(-) embryos
	UM	9.4 (±1.1)
	SO	3.3 (±4.5)*
	PA	3.1 (±4.0)*

*significantly less than UM, p ≤ 0.07

Within this research progesterone antagonist administration following the transfer of E3.5(-) embryos into R2.5 recipient mice, which in theory would delay the development of recipient endometrium and thus increase the time available for implantation-competency, did not demonstrate increased mean implantation rates (Gao, et al., 2001; Huang, et al., 2005; Table 63).   In contrast, low levels of implantation were observed (3.1 implants per recipient mouse), which was comparable to those observed within the literature (Liu, et al., 2003; Liu, et al., 2008). This raised the questions surrounding progesterone antagonist's mechanism of action. Possible mechanisms included: delayed pinopode development; reduced embryo apposition or invasion (Dai, et al., 2003; Goa, et al., 2001; Sarantis, et al., 1988; Song & Chen, 2007).

A small number of studies, which asynchronously transferred E3.5 embryos into recipient mice, whose endometrium was very immature (R0.5 and R1.5), also resulted in low implantation rates (Doyle, et al., 1963; Goto, et al., 1993; Nakayama, et al., 1995; Wakuda, et al., 1999). Similar to the administration of RU486, a progesterone antagonist, on day-3.5 pc, 24 hours following ET, transferred embryos had more than sufficient time to reach implantation-competency and implant.   Outside of the

premature uterine exposure of transferred donor embryos, no other manipulations were performed. For this reason, it was possible that delayed pinopode development, or reduced embryo apposition or invasion, were not the only mechanisms of action potentially responsible for low implantation rates subsequent to progesterone antagonist administration.

Findings in context of the literature, suggested that a very immature endometrium might not support embryo growth and development. Further experimentation would be required to confirm this statement and could include the transfer of either E3.5(-) or E4.5(-) embryos into R1.5 recipient mice. Such experimental design was attractive due to the simple strategy of asynchronous ET. In addition, the technique was not associated with multiple mechanisms of action and was more suitable to explore the impact of a delayed endometrium on mean implantation rates. Progesterone antagonist administration subsequent to ET was, therefore, not considered suitable to explore the time required or available for implantation-competency on mean implantation rates.

The combined influence of the time required and available for embryo implantation-competency on mean implantation rates was comparable to McLaren and Michie (1955), who also demonstrated a combined influence on implantation rates (Table 64). Although no other studies explored both the time required and available for embryo implantation-competency on implantations rates, a number of studies were able to demonstrate the impact the time available for embryo implantation-competency on implantation rates (Ertzeid & Storeng, 2001; Fossum, et al., 1989; Kelley, et al., 2006; Paria, et al., 1993; Rülicke, et al., 2006; Figure 90). Therefore, the current research was able to confirm the influence of both the time required and available for embryo implantation-competency and thus the importance of considering both times when targeting a certain level of implantation, necessary for *in vivo* implantation murine model standardization.

Table 64 Implantation frequencies demonstrated by McLaren and Michie (1955) following the transfer of donor embryos into naturally-pregnant recipient mice.

		Embryo developmental age	
		E2.5	E3.5
Recipient developmental age	R2.5	11%	23%
	R3.5	0%	11%
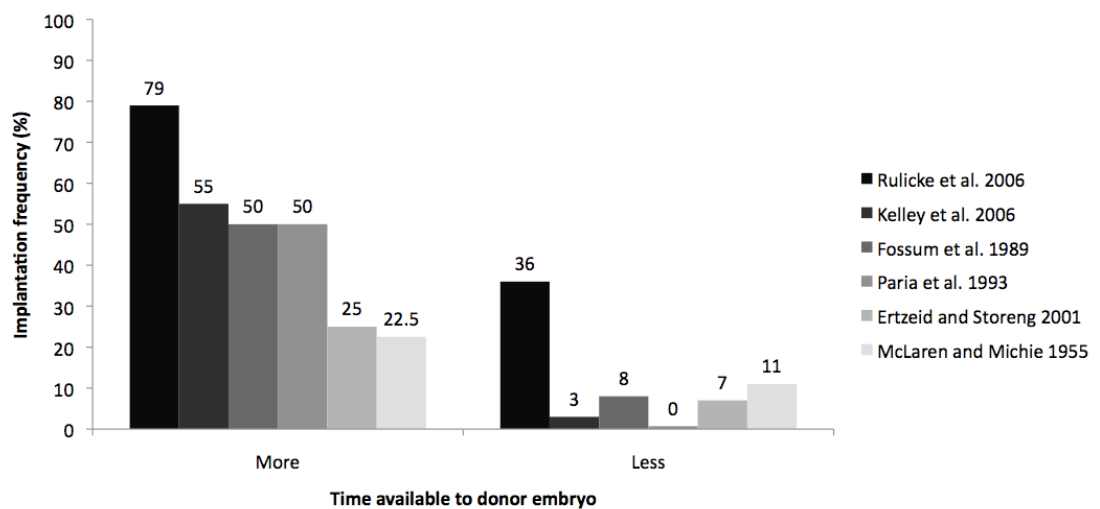
Figure 90 Impact of the time available for embryo implantation-competency on implantation rates found within the literature. Data includes studies that utilised synchronous and asynchronous ET, and ET into SO-recipient mice.

6.1.2 Inconsistent implantation rates were observed within models that achieved low levels of implantation

Although a clear pattern was observed in regard to mean implantation rates, individual implantation rates within models that achieved low levels of implantation were inconsistent. Figure 91 demonstrates substantial mouse-to-mouse variability within models, where some recipient mice achieved zero implantation. Utilizing such inconsistent suboptimal models to determine the potential of embryo manipulation, as an IVF-intervention, was troubling. Inconsistent implantation rates within models could potentially translate to incomparable uterine environments and opportunity to implant. As a result, any subtle but significant improvements in suboptimal implantation rates, as a consequence of embryo manipulation, could be masked.

None of four models that exhibited suboptimal levels of implantation achieved consistent mean implantation rates (Figure 91). As specified by the selection criteria for the most suitable suboptimal model, mean implantation rates would need to vary by only ± 1 implant. In contrast, implantation rates varied from ± 4 – 7 implants per recipient mouse, with a large proportion of recipient mice exhibiting zero implantation. As a result of this mouse-to-mouse variability, both suboptimal and optimal levels of implantation were demonstrated within models. For this reason, it could not be ensured that embryos, which were transferred into any one type of model, were exposed to comparable uterine environments mouse-to-mouse. Hence, the ability of the model to demonstrate any subtle, yet significant consequences of an IVF-intervention, would be diminished.

The literature revealed little insight into the mouse-to-mouse variability observed within each model. In fact many gaps in knowledge surfaced, which surrounded both the beginning of endometrial receptivity and how altered embryo-endometrial developmental synchrony influenced it's timing. Therefore, this research methodically explored the beginning of endometrial receptivity within natural mouse pregnancy, and synchronous and asynchronous ET (Chapter 5). The significance of these findings is discussed in Section 6.2.

Panel A – ET into superovulated recipient mice

Panel B – Progesterone antagonist administration following ET into the Optimal Model

Panel C – Synchronous transfer of E3.5(-) embryos into R3.5 recipient mice

Panel D – Asynchronous transfer of E4.5(-) embryos into R3.5 recipient mice
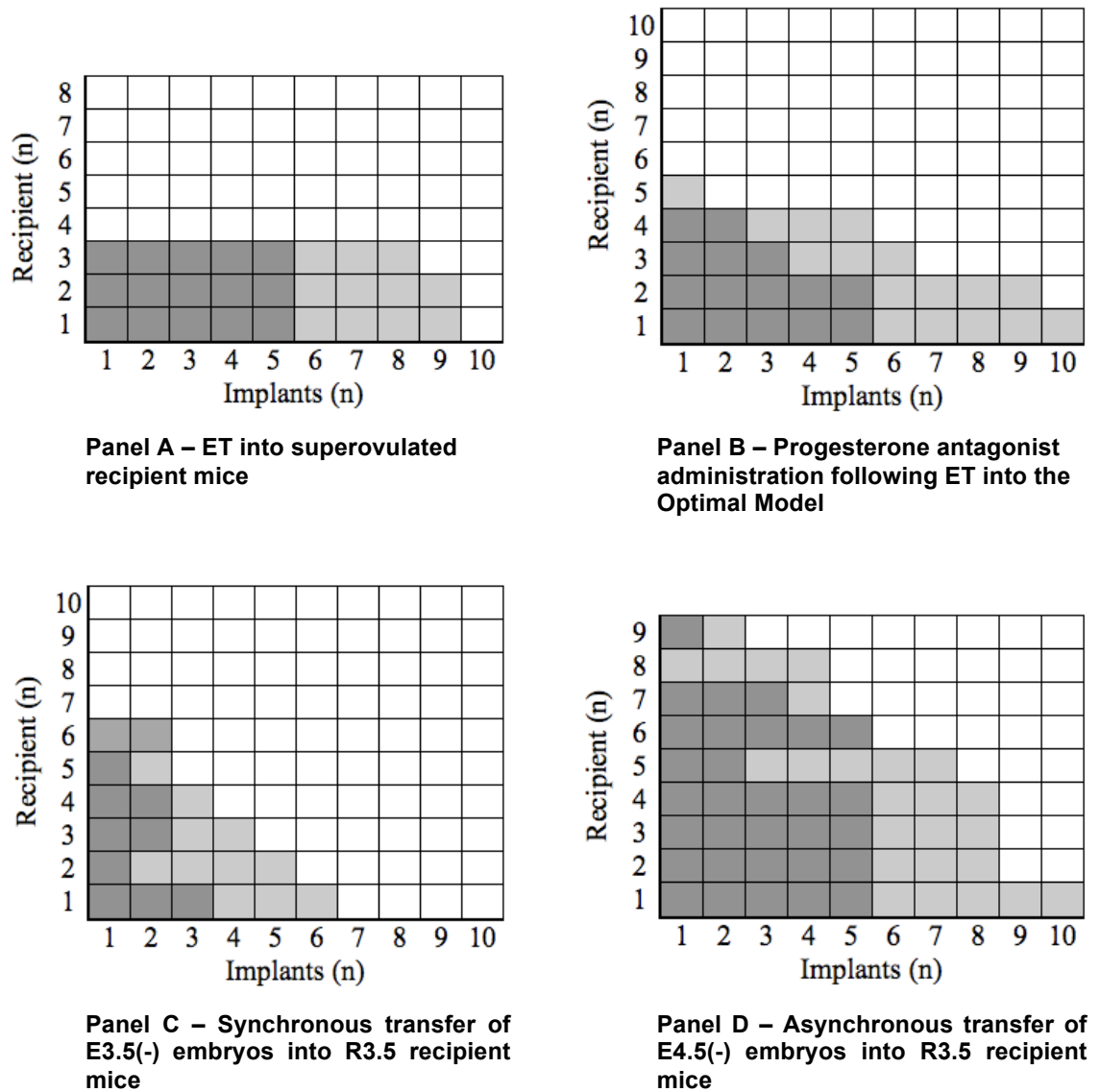
Figure 91 Numbers and location of implants day-17 pc following ET into a selection of suboptimal models. Embryo transfer into SO-recipient mice exhibited a mean of 3.3 implants per recipient mouse, ± 6 implants (Panel A; Figure 25). Progesterone antagonist administration following ET achieved a mean of 3.1 implants per recipient mouse, ± 7 implants (Panel B; Figure 31). The synchronous and asynchronous transfer of E3.5(-) or E4.5(-) embryos into R3.5 recipient mice demonstrated means of 2.2 and 6.2 implants per recipient mouse, where both varied by ± 4 implants (Panels C and D; Figures 34 and 37). No significant difference between left and right uterine horns in Panel D (p = 0.893). Features as per Figure 20.

6.1.3 The Optimal Model's ability to detect negative consequences of manipulated embryos

This research indicated that the Optimal Model held the ability to detect negative consequences of manipulated embryos. This included both pre-implantation (reduced percentage implantation frequency) and post-implantation losses (reduced viability). For this reason, the Optimal Model could be used to determine the safety of embryo manipulation as an IVF-intervention, and thus could be considered as standard.

Within this research, the asynchronous transfer of E3.5(-) embryos into R2.5 recipient mice was chosen as the Optimal Model. Two criteria served as a basis for this selection. These were: 1) for every 10 embryos transferred, the mean implantation rate would be comparable or greater than natural mouse pregnancy (9.0 implants per mouse ± 1 implant); and 2) a minimum of 70% implants would need to be viable. The transfer of E3.5(-) donor embryos into R2.5 recipient mice met both criteria, achieving a mean of 9.4 implants per recipient mouse, with 74% of those implants considered viable. This ensured that the Optimal Model did not have any adverse effects on implantation itself and that sufficient numbers of fetuses would be available to assess fetal and placental weights, gross morphology and development. Thus, experimental numbers would be kept to a minimum.

Before the Optimal Model could be used to determine the safety of embryo manipulation, it was important to confirm it held an ability to detect negative consequences of manipulated embryos. If the model's ability remained unconfirmed, the potential impact of misleading data would be unknown and could mask any negative consequences of embryo manipulation. Five different groups of manipulated embryos were transferred into the Optimal Model alongside unmanipulated embryos (control). In context of the current experimental design, two groups, FSL-HA$_{high}$ and BioG manipulated embryos, successfully demonstrated the Optimal Model's ability to detect negative consequences of manipulated embryos, pre and post-implantation. These included a reduced percentage implantation frequency and fetal weight. Consequently, the Optimal Model was considered suitable to explore the safety of embryo manipulation.

Due to the vast numbers of variables between similar *in vivo* implantation murine models, it was difficult to draw any direct comparison between this research and the literature. As indicated in Table 65, Lim, et al. (2006) retrieved embryos from adult donor mice, which is in contrast to this research, where prepubescent donor mice were

utilised. In addition, the day of embryo retrieval differed, day-3.5 pc verses day-1.5 pc, which was used within this research.   As a consequence, donor embryos were exposed to very different uterine environments prior to retrieval, which could have had substantial impact on embryo growth, development and implantation (Wakuda, et al., 1999; Wang & Dey, 2006).

An additional variable to consider between similar *in vivo* implantation murine models was the substantially lower implantation frequencies (22 – 68%) compared to this research, which achieved 95% implantation (Table 26). Such high frequencies were previously considered unattainable. This was due to the highly technical nature of ET, in context of expertise and equipment quality; and the procedures which donor generated embryos and recipient mice, not to mention the age of experimental mice. As demonstrated in Table 65, the mouse strain and the numbers of embryos transferred could also influence implantation rates between different research groups. A further variable, which could account for the difficult comparison between the literature and this research, was the fact that research groups, who explored embryo manipulation, often focused on improving implantation rates rather than investigating potential negative consequences (Lane & Gardner, 1994; Lim, et al., 2006). Collectively, all points are indicative of the incomparable uterine environment, and/or the manipulation embryos were exposed to within models found within the literature. Therefore, comparison between the literature and this research was difficult.

Table 65 Embryo transfer outcomes and experimental variables associated with studies thought to be comparable to the Optimal Model. Studies shared comparable donor embryo and recipient mouse endometrium developmental age of Optimal Model. Key: prepub, prepubescent; SO, superovulated; N, naturally ovulated; and U, urinary.

Researcher	Recipient mouse strain	Implantation rate (%)	Viability rate (%)	Recipient number	Time of assessment (days pc)	Embryo donor mouse age	Naturally or superovulated	Urinary or recombinant gonadotropin	Day of embryo retrieval (pc)	Embryo culture status	Number of embryos transferred per recipient	Number of embryos transferred per uterine horn	Unilateral or dual horn transfer
McLaren & Michie, 1955	C3H x C57BL	22	-	15	16.5	prepub	SO	U	3.5	*in vivo*	mean of 9	-	Unilateral
Lim, et al., 2006	ICR	26	-	13	4.5	adult	SO	U	2.5	*in vitro*	20	10	Dual horn
Wakuda, et al., 1999	ICR	52	-	20	11	adult	SO	U	3.5	*in vivo*	6	3	Dual horn
Lane & Gardner, 1994	C57BL/6 x CBA/Ca	52	36	5 – 6	15	prepub	SO	U	0.5	*in vitro*	-	-	n/a
Mullen & Carter, 1973	C57BL/10GnDg	65	49	12	16 - 17	-	N	-	2.5	*in vitro*	8 -14	-	Dual horn
Mullen & Carter, 1973	(C57BL/10 GnDg x CBA/J) F$_1$	72	65	12	16 - 17	-	N	-	2.5	*in vitro*	8 - 14	-	Dual horn
Doyle, et al., 1963	BALB/C x C57BL	-	43	11	> 18	adult	N	-	3.5	*in vivo*	-	-	Dual horn
Nakayama, et al., 1995	ICR	-	64	46	10	prepub	SO	U	3.5	*in vivo*	2	-	-
Wiebold & Anderson, 1986	-	-	66	-	> 18	-	-	-	-	*in vitro*	-	-	-
This study	B6CBA1/J	95	70	13	17	prepub	SO	U	1.5	*in vitro*	10	5	Dual horn

Storeng and Jonsen (1984), who also transferred E3.5 embryos into R2.5 recipient mice, did provide some comparison. Their focus had been to determine the recovery of embryos after in vitro exposure to $NiCl_2.6H_2O$ rather than their model's ability to detect negative consequences of manipulated embryos. Yet, an increase in fetal non-viability from 12% (unmanipulated) to 20% (NiCl2.6H2O manipulated) following ET indicated their model was able to detect negative consequences of manipulated embryos ($p < 0.05$, calculated by Storeng & Jonsen, 1984). Therefore, this research and the findings of Storeng and Jonsen (1984) strongly support the use of the Optimal Model as a way to determine the safety of embryo manipulation.

6.1.4 The Suboptimal Model's ability to detect positive consequences of manipulated embryos

This research demonstrated that embryo transfer media, which contained free or inserted-HA, was unsuitable to verify the Suboptimal Model's ability to detect positive consequences of manipulated embryos, an IVF-intervention. To verify this ability further investigation would be required with a molecule, which could induce improvement. Only once this ability was confirmed could the Suboptimal Model be used to determine the potential embryo manipulation held in improving suboptimal implantation rates.

The asynchronous transfer of E4.5(-) donor embryos into R3.5 recipient mice was selected as the Suboptimal Model within this research. Two criteria, which formed the basis for selection, were met in part. These included: 1) the models design needed to prevent implantation (increased pre-implantation loss) rather than disrupt or damage implantation (increased post-implantation loss); and 2) resulting mean implantation rates would be approximately 6 implants per recipient mouse for every 10 embryos transferred (± 1 implant). Although the Suboptimal Model exhibited a mean of 6.2 implants per recipient mouse, implantation rates varied by up to ± 4 implants per recipient mouse, which did not meet the preferred ± 1 implant. Nonetheless, those criteria that were met ensured that the model did not have an adverse affect on post-implantation development, exhibited the capability of implantation, but also would be able to demonstrate an increase in implantation rates.

Unexpectedly, implantation frequencies did not increase following FSL-HA$_{high}$ or FSL-HA$_{mid}$ (inserted-HA) embryo manipulation or through UTM™ transfer media (free-HA). Thus, data suggested that the suboptimal model might not hold the ability to detect positive consequences of manipulated embryos. However, it was important to

determine that the findings were not masked by an inability of inserted or free-HA to improve implantation rates within the suboptimal model.

An attempt was therefore made to replicate original data, which was instrumental in the commercial introduction of HA-containing IVF transfer medium. In spite of this, free-HA containing media demonstrated no significant improvement in implantation frequency following ET (52%, free-HA; 56%, media alone). In contrast, implantation frequencies within Gardner et al. (1999) improved dramatically from 48% (media alone) to 80% (free-HA). Therefore, data indicated that inserted or free-HA may not hold the ability to improve implantation rates within the Suboptimal Model. In order to confirm this theory, the differing variables found between each study were examined.

At the time of transfer, donor embryo (E4.5[-]) and recipient mouse endometrium (R3.5) developmental age were comparable between both studies. There was, however, a strain difference between donor embryos sources. Within this research, donor embryos were sourced from prepubescent B6CBAF1/J mice. In contrast, Gardner et al. (1999) retrieved donor embryos from CF-1 mice. Although the superovulation protocol and drug source were comparable, a lack of clarity surrounded donor mice age within the Gardner et al. study (1999). Nonetheless, Williams (2008), and Gardner et al. (1999) were able to demonstrate comparable numbers of day-4.5 pc blastomeres between the two species (B6CBAF1/J, 56 ±5.9; CF-1, 60 ±3.5). Implantation frequencies were also comparable (B6CBAF1/J, 56%; CF-1, 56%). Therefore, different strain sources of donor embryo were not thought to contribute towards the conflicting data.

It was also unlikely that a difference in HA-diluent led to conflicted data. Within both studies, embryo culture media was used as a HA-diluent. Within this research, media contained macromolecules derived from bovine serum albumin (BSA). In contrast, Gardner et al. (1999) utilised media that did not contain any macromolecule. However, comparable embryo development, implantation frequencies and fetal viability between media containing BSA and no macromolecule were demonstrated (Gardner, et al., 1999). By day-4.5 pc, 71% and 68% of embryos cultured in media containing BSA or no macromolecule had become blastocyst embryos (p < 0.05, calculated by Gardner et al., 1999). Fifty-three percent of embryos cultured and transferred in BSA-containing media resulted in implants, which was comparable to those embryos associated with no macromolecule (56%, p < 0.05, calculated by Gardner et al., 1999). Of those embryos, 36% and 44% respectively, resulted in viable fetuses (p < 0.05, calculated by

Gardner et al., 1999). For this reason, a difference in HA-diluent was unlikely to contribute towards the conflicting data.

Within this research, experiments attempting to replicate the findings of Gardner et al. (1999; Section 4.6.4) utilised *Streptococcus equi*, with a 1.5 - 1.8 x 10^6 Da molecular weight range, as the source of free-HA. In contrast, Gardner utilised free-HA from Rooster comb (Pharmica, Sweden), which had an average molecular weight of 3.0 x 10^6 Da. However, the commercially available Embryoglue®, which resulted from Gardner et al. (1999), shared a comparable HA source with this research (*Streptococcus equi*). While the average molecular weight of Embryoglue® (2.3 x 10^6 Da) was greater than the free-HA source utilised in Section 4.6.4, it fell within the molecular weight range of UTM™ transfer media (2.4 - 3.6 x 10^6 Da; Medicult, Denmark). UTM™ transfer media was utilised as the free-HA source compared with inserted-HA within Section 4.6.1, and was also unable to improve suboptimal implantation rates. Therefore, it was unlikely that a difference in HA source, and consequently differing molecular weights, contributed towards conflicting data.

The incubation time of free-HA within this research (two hours) exceeded the time utilised by Gardner et al. (1999), by up to 25 minutes. Nonetheless, Gardner et al. (1999) demonstrated normal blastomere numbers up to 4.5-days of free-HA embryo incubation. By this time, embryos cultured in free-HA containing media contained on average 64 ±3.6 blastomeres (Gardner, et al., 1999). In contrast, those embryos cultured in media containing BSA resulted in comparable mean numbers of blastomeres (60 ±3.6; $p < 0.05$ calculated by Gardner et al., 1999). Williams was also able to demonstrate normal blastomere numbers and percentage blastocyst formation following FSL-HA$_{high}$ and FSL-HA$_{mid}$ manipulation of embryos (2008). Following FSL-HA$_{high}$ manipulation at 1mg/mL for two hours, embryos contained 101 ±9.5 blastomeres, which was comparable to unmanipulated embryos (108 ±9.5; $p > 0.1$). Subsequent to 18 hours of FSL-HA$_{mid}$ manipulation at 2mg/mL, which was 16 hours longer than this research, 77% of embryos developed into blastocysts, which was comparable to unmanipulated embryos (76%; $p > 0.05$). Therefore, an extended period of free-HA incubation prior to ET was unlikely to be detrimental towards implantation rates, masking the ability of HA to improve those implantation rates.

Turning to other previous research, variable implantation rates associated with free-HA containing transfer media have also been reported (Loutradi, et al., 2007; Loutradi, et al., 2008; Mahani & Davar, 2007; Simon, et al., 2003; Valojerdi, et al., 2006).

Nevertheless, a recent Cochran systematic review was able to confirm a small, but significant increase in implantation and pregnancy rates as a result of HA-containing transfer media (Bontekoe, et al., 2010). Meta-analysis of 13 randomised controlled human studies investigating the benefits of HA, with a total of 3252 participants, led to their conclusions. Their data suggested that HA was able to facilitate implantation outside of the realms of improved adherence (Bontekoe, et al., 2010). Therefore, it is possible that the impact of inserted or free-HA within the Suboptimal Model was obscured in some way.

Experimental features not normally published in peer reviewed journal articles, could account for the differing results. These included: 1) operator skill level; and 2) quality of surgical equipment. Operator skill and consistency is well known to infleuce ET outcomes (Hogan, et al., 1994). In addition, it is unknown within Gardner et al. (1999) whether more than one operator was used. If this were the case, an additional layer of variability could have been present. Furthermore, quality of surgical equipment can influence ET outcomes. An example is seen the pioneering work of McLaren and Michie (1955), who utilised hypodermic needles, with a 0.47 mm outer diameter, to puncture uterine horns prior to transfer. In contrast, 29 G needles utilised in this research had an outer diameter of 0.34 mm. McLaren and Michie (1955) suggested that their low implantation rates resulted surgical interference, which could include escape of embryos through the entry point created by the larger needle diameter.

An addition possibility surrounded treatment allocation to uterine horns. Within Gardner et al. (1999), allocation of treatments was randomized to each uterine horn. This included the control group (no-macomolecule). As a result, one recipient mouse could receive either two groups of the same treatment, two different treatment groups, one treatment and one control group, or two groups of controls. Matorras et al. (2005) and Ye (2006) have demonstrated through mathematical modelling that with every successful embryo implantation, the implantation probability of subsequent embryos increases. Therefore, if one treatment promotes implantation more than another, how it is paired with another treatment or control group can influence the overall implantation opportunity within that one recipient mouse. In contrast, within this research, each recipient mouse received both treatment and control embryos (side and order assigned at random). For this reason, mice could act as their own control, thus reducing experimental variability between mice and enabling removal of recipient mice that did not result in implants.

Outside experimental design and technique, it is possible that the high molecular redundancy associated with murine implantation could have obscured findings (Aplin, 1997; Tranguch, et al., 2005). Alternatively, a positive impact of HA on human implantation rates may not translate to improved murine implantation rates. Differences in physiology and fecundity could account for a lack of translation (Carson, et al., 2000; Chavatte-Palmer and Guillomot, 2007; Lee and DeMayo, 2004). As a result, either form of HA was unsuitable to determine the Suboptimal Model's ability to detect positive consequences of manipulated embryos. Further investigation would be required prior to using the Suboptimal Model to determine the potential embryo manipulation had to improve implantation rates.

Manipulated embryos that mimicked the surface expression profile of implantation-competent embryos may offer an opportunity to assess the Suboptimal Model's ability to detect positive consequences of manipulated embryos. As suggested in Section 6.2.3, such manipulation may increase embryo-endometrial cross-talk, inducing early endometrial receptivity and increasing implantation opportunity. Firstly, it would be necessary to identify and manufacture suitable manipulation molecules. Followed by assessment of their safety *in vitro* and *in vivo*, and ability to improve *in vitro* attachment. Subsequently, potential molecules could determine the Suboptimal Model's ability to detect positive consequences of manipulated embryos. Investigations, such as these, would have taken an excess of six months, which exceeded the scope of this research.

6.2 Refinement of in vivo implantation murine models

Experiments exploring the beginning of endometrial receptivity were able to extend current knowledge of *in vivo* implantation murine model standardization in three ways. The first point surrounded specific advice on how to achieve greater model uniformity, where implantation rate consistency within models that achieved low levels of implantation, was dependent on the time of coitus, ET, and autopsy, in terms of hours not days. Experiments surrounding the beginning of endometrial receptivity, which strictly controlled the time of these variables, provided direct evidence of improved implantation consistency. The subsequent two points surrounded basic fundamental knowledge of *in vivo* implantation murine modelling, which provided insight into the mechanism of action behind specific levels of implantation. These included: 1) naturally pregnant mouse endometrium was receptive by 88 hours pc, four hours

earlier than previously reported; and 2) the premature uterine presence of mature pre-implantation embryos within recipient mice, whose endometrium would not normally be exposed to embryos at that time, could induce early endometrial receptivity following uterine ET. The following sections will discuss these findings and their significance.

6.2.1 Implantation rate consistency dependent on the distinct moments of coitus, ET and autopsy

This is the first report to identify the impact of distinct moments of coitus, ET, and autopsy, on implantation rate consistency, in terms of hours not days. While the time of coitus, ET and autopsy had little impact within murine models that exhibited high levels of implantation, within those models that demonstrated low levels of implantation, significant influence was demonstrated. Such implantation rate inconsistency was observed both on day-17 (baseline, Chapter 4) and 3.5 pc (beginning of endometrial receptivity, Chapter 5). Utilizing models that presented inconsistent implantation rates would risk masking subtle, but significant data, and could lead to difficulties in comparing experimental findings with other research groups. Therefore, identifying the impact of the distinct moments of coitus, ET and autopsy was highly advantageous in developing standardised *in vivo* implantation murine models.

The beginning of endometrial receptivity was investigated within synchronous and asynchronous ET by assessing for the presence of implants at 88, 90 and 92 hours pc (day-3.5 pc). Such time-points were selected in an attempt to span both the pre-receptive and receptive endometrial phases. Mean implantation rates and grading of blue-bands at each assessed time-point identified the presence endometrial receptivity, indicated implantation advancement, and thus suggested where in the endometrial phase the time-point may have been located. Additionally, individual implantation rates within models that achieved low levels of implantation, demonstrated mouse-to-mouse variability at each time-point. Due to the strict control of variables within experiments exploring the beginning of endometrial receptivity, data was able to suggest a varied time of coitus, ET and autopsy for being responsible for the observed implantation rate inconsistencies throughout this research (days-3.5 and 17 pc). In addition, the distinct moments of coitus are dependent on whether female mice have successfully ovulated (Hogan, et al., 1994). Thus, through its influence on the timing of coitus, ovulation could also indirectly influence implantation rate consistency. A critical

analysis within the subsequent paragraphs will serve to explain how these variables were identified.

Three potential factors, which may have contributed towards the inconsistent implantation rates observed, received careful consideration. These were: 1) an inability to allow or support implantation within recipient mice; 2) a variable time required by donor embryos to become implantation-competent and implant; and 3) a variable time available for embryo implantation-competency and implantation.

The first of these potential factors, an inability to allow or support implantation within recipient mice, may have accounted for the high proportion of mice that exhibited zero implantation. However, experiments that explored the beginning of murine endometrial receptivity on day-3.5 pc of natural and pseudo-pregnancy suggested an inability to allow or support implantation held little influence on consistency of implantation rates. Zero implantation was observed at 90 hours pc (day-3.5 pc) within a proportion of naturally pregnant (75%) and R3.5 pseudo-pregnant recipient mice that received E4.5(-) embryos (50%). In contrast, by 92 hours pc, all recipient mice within each group, demonstrated comparable numbers of implants to expected pregnancy rates on day-17 pc (baselines). If inability to allow or support implantation was a potential contributing factor, zero implantation within mice would have been observed right through to day-17 pc. Thus, data indicated that an inability to allow or support implantation was unlikely to contribute towards the inconsistencies observed in implantation rates.

The second potential factor to consider was a variable time required to reach embryo implantation-competency, which had been shown to influence mean implantation rates within Chapter 4. While such a factor did not hold any concern within natural mouse pregnancy, its influence needed to be considered within models utilizing ET. When a pool of donor mice are used to retrieve embryos, a natural variation in the time required for implantation-competency is inevitable. This is due to varied times of coitus between individual donor mice. There is a risk that the time required by donor embryos to become implantation competent could vary mouse-to-mouse. In some cases, this time required may exceed what is available and result in zero implantation.

Within this research, however, recipient mice received embryos from a pool of donor mice. For this reason, the time required for implantation-competency and implantation would be comparable between groups of transferred embryos. In addition, the majority

of transferred embryos were high quality (A-grade), and any small variation in their stage of development did not influence implantation rates. It is worth noting, however, that the introduction of IVF or intracytoplasmic sperm injection could also eliminate the natural variation in the time required for implantation-competency. If time and cost were not limiting factors, these techniques could be utilised in future research.

The third potential factor to consider was a varied time available for embryo implantation competency and implantation, which was also shown to influence mean implantation rates within Chapter 4. Such a factor was dependent on the distinct moments of coitus, ET and autopsy. Within experiments that explored the beginning of endometrial receptivity, the times of ET and autopsy were standardised. However, the time of coitus was only partially restricted. The time of first male-female contact was only allowed from the mid-point of the dark phase, the period of time the mouse colony environment was not exposed to light. Under normal conditions, coitus is thought to occur at approximately this time, and will only occur if female mice have successfully ovulated (Hogan, et al., 1994). Eight to ten hours after first male-female contact, female mice within this research were assessed for the presence of seminal plugs, indicative of pregnancy or pseudo-pregnancy (Hogan, et al., 1994). For that reason, recipient endometrial development age at the time of ET could be equal to or less than real-time pc. Yet, how this translated to the length of time available for embryo implantation-competency and implantation opportunity was dependent on the time of autopsy.

Figure 92 illustrates how a varied time of coitus influences the length of time available for embryo implantation-competency and implantation when autopsies are performed on day-3.5 pc (beginning of endometrial receptivity). As indicated within Schematic A of Figure 92, when the times of first male-female contact and coitus were comparable, endometrial developmental age would be comparable to real-time pc. In contrast, if coitus occurred at a later time to first male-female contact, endometrial developmental age would be immature compared to real-time pc (Schematic B, Figure 92). Therefore, in the event of zero implantation, it is plausible that recipient mouse endometrium had not yet become receptive by the time of autopsy. Thus, there would be no opportunity to implant.

In cases where the receptive mouse endometrium is able to become receptive by the time of autopsy, but is still immature compared to real-time pc, the level of implantation would be dependent on the proportion of donor embryos that are able to become fully

implantation-competent and implant by that time. Individual mice within each assessed time-point can therefore experience varied endometrial maturity and thus a varied time available for embryo implantation-competency and implantation, exhibited by inconsistent implantation rates.

Within experiments where the time of autopsy was located well after the completion of endometrial receptivity, such as day-17 pc (baselines), both the time of coitus and ET had the potential to influence the time available for embryo implantation-competency and implantation. While the real-time recipient endometrium developmental age, in terms of days pc, was comparable within models, the specific time of ET on that day, in terms of hours pc, was not standardised. Therefore, the distinct moments of coitus and ET potentially influenced the length of time available for embryo implantation-competency and implantation.

Imagine three different embryo transfers were performed on the same day (Figure 93). Recipient A and B both experienced coitus shortly after the first point of male-female mouse contact. However, the ET into recipient B was performed much later in the day compared to recipient A. As a consequence donor embryos within recipient B had less time available to reach implantation-competency and implant. In contrast, recipient C experienced coitus well after the first point of male-female contact. As a result, the endometrium was immature for its real-time age. Therefore, if the ET into recipient C was also performed much later in the day, the time available for embryos to become implantation-competent and implant would be comparable to recipient A. For this reason, it was most likely that a varied time of coitus and ET were responsible for a varied time available for embryo implantation-competency, resulting in inconsistent implantation rates on day day-17 pc (baselines).
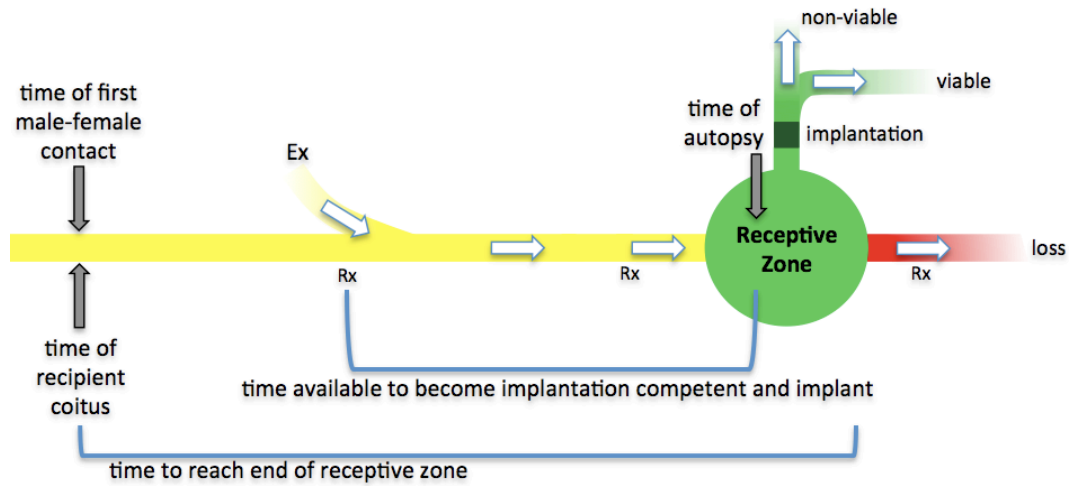
Schematic A – endometrial developmental age at time of autopsy comparable to real-time
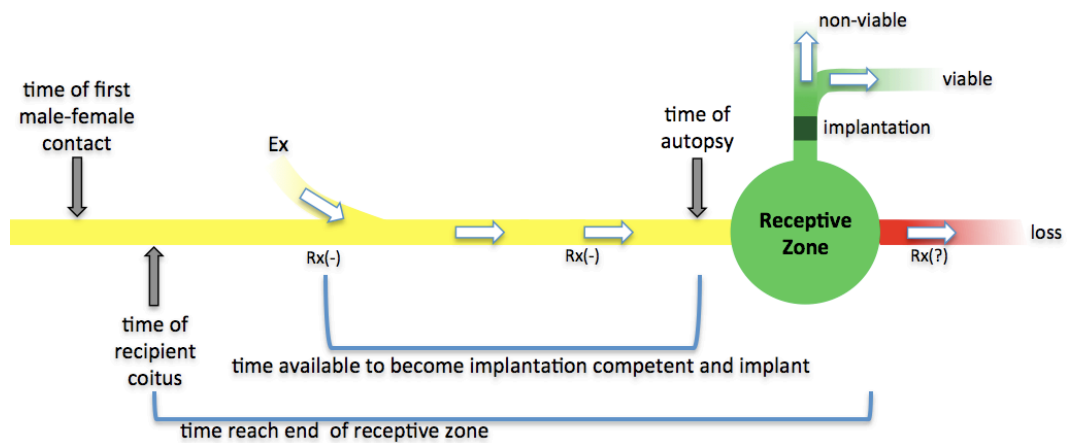
Schematic B – endometrial developmental age at time of autopsy immature compared to real-time.

Figure 92 Schematic representation illustrating the impact of varied times of coitus within experiments identifying the beginning of endometrial receptivity. Schematic A illustrates that when the time of coitus is comparable to first male-female contact, endometrial developmental age at the time of autopsy is comparable to real-time. Schematic B illustrates that when the time of coitus is incomparable, the endometrium is immature compared to real-time age at the time of autopsy. Less time is then available for donor embryos to become implantation-competent and implant, and can lead to zero implantation opportunity.
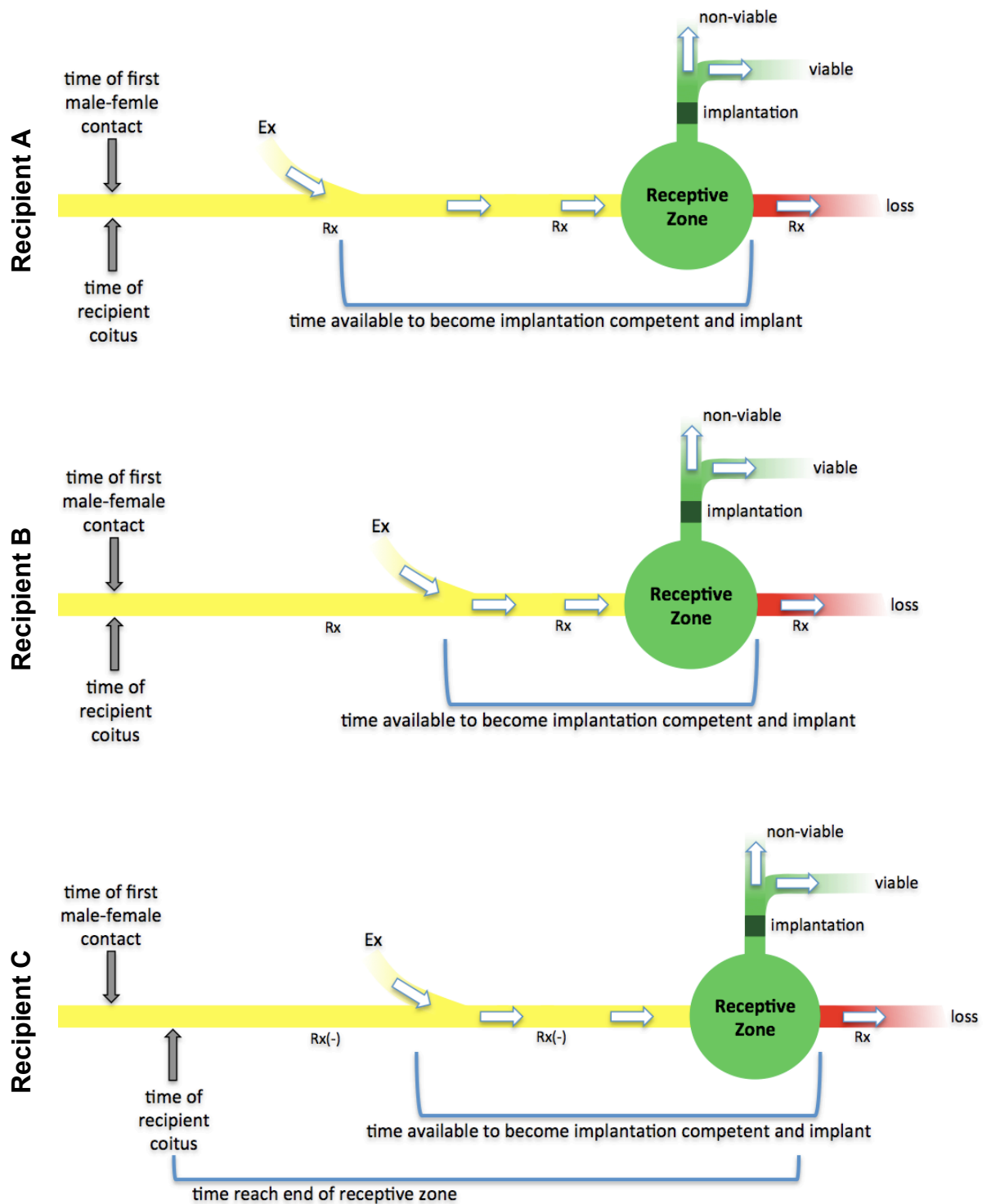
Figure 93 Schematic representation illustrating the impact of varied times of coitus and ET within experiments investigating model baselines. The time of first male-female contact and coitus are comparable within Recipients A and B. Due to a later time of ET, Recipient B has less time available for embryos to reach implantation-competency and implant compared to Recipient A. The time of coitus is later than first male-female contact in Recipient C. Yet, the time of ET is comparable to Recipient B. The length of time available for embryos to reach implantation-competency and implant in Recipient C is then comparable to Recipient A.

The experimental design utilised to explore the beginning of endometrial receptivity provided a direct example of how the strict control of the time of coitus, ET and autopsy can improve implantation rate consistency. For that reason, adopting such experimental design could provide a sound alternative. The time of ET and autopsy were already strictly controlled, and were shown to improve implantation rate consistency. For example, following the transfer of E4.5(-) embryos into R3.5 recipient mice, numbers of implants per recipient mouse (mean of 6.2 implants per recipient mouse) varied up to ± 4 implants on day-17 pc. In contrast, numbers of implants varied only up to ± 2 implants at 92 hours pc (day-3.5 pc; mean of 3.3 implants per recipient mouse).

If combined with a standardised time of coitus and locating the time of autopsy closer to the end of endometrial receptivity, a mean implantation rate of 6 implants per recipient mouse ± 1 implant, the second selection criterion for the most suitable suboptimal model, could be achieved. For example, the opportunity for coitus could be reduced to two hours compared to the 8 – 10 hours within this research, which could minimise mouse-to-mouse variability considerably. Although, such experimental design would require thorough investigation prior to its use, findings of this research point towards significant opportunities to simply and effectively improve model consistency. It is worth noting that any potential improvement could be masked by variable technical skill. Only those operators with a consistent skill level, albeit low or high, could observe any benefit in finely controlling the times of coitus, ET and autopsy. Nonetheless, implementation of findings could bring the development of standardised *in vivo* implantation murine models a step closer.

6.2.2 Mouse endometrium can be receptive by 88 hours pc

Evidence of murine endometrial receptivity within this research has extended beyond previous literature, which did not report receptivity within natural mouse pregnancy prior to 92 hours pc (Finn & McLaren, 1967; McMaster, et al., 1993). Within this research, implants were detected as early as 88 hours pc, which suggested murine endometrium could receptive by this time, four hours earlier than previously reported (Figure 94).

In fact, no zero baseline mean implantation rates were achieved within this research. In particular, R2.5 recipient mice, that received E3.5(-) or E4.5(-) embryos, demonstrated fully saturated mean implantation rates by 88 hours pc (8.4 and 7.8

implants per recipient mouse, Table 54 and Table 56). Like in natural mouse pregnancy, there is an inherent, yet small variation in time required for implantation-competency when pools of donor embryos are utilised for transfer. For this reason, such high levels of implantation, which were comparable to their corresponding baselines on day-17 pc (9.4 and 9.8 implants per recipient mouse, $p \geq 0.151$; Table 26 and Table 28), did not reflect the lower levels of implantation expected at the beginning of endometrial receptivity. Data therefore indicated that within the context of the asynchronous uterine transfer of embryos into R2.5 recipient mice, implantation and the corresponding endometrial receptivity most likely began prior to 88 hours pc. Further experimentation, which would include a series of time-points prior to 88 hours pc, was necessary to identify zero baseline mean implantation rates and thus the beginning of endometrial receptivity. Chapter 8 will serve to discuss such experimental potential. In addition, the significance of saturated mean implantation rates observed by 88 hours pc is discussed in greater depth within Section 6.2.3.
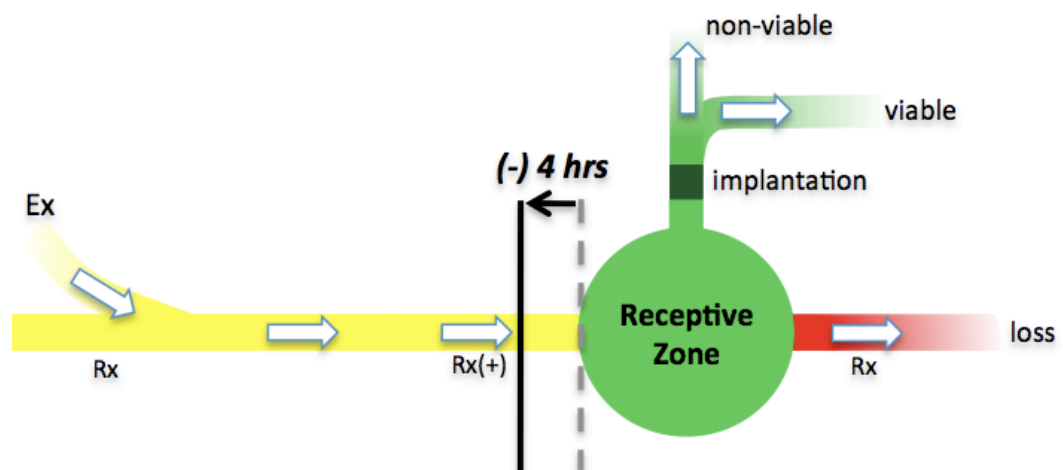
Figure 94 Schematic representation illustrating murine endometrial receptivity four hours earlier than the literature. This research demonstrated endometrial receptivity by 88 hours pc. The earliest known report of endometrial receptivity in the literature was 92 hours pc.

A four-hour difference in the earliest indication of murine endometrial receptivity was a significant finding given that the entire length of the endometrial phase was thought to be at least 12 hours long, but less than 24 hours (Doyle, et al. 1963; Ma, et al., 2003; Paria, et al., 1993; Psychoyos, 1973a, 1973b; Yoshinaga, 1988). Such a difference could translate to a 33% increase in the length of time murine endometrium was

receptive. Thus, data suggested implantation opportunity could be greater than first thought. As experiments focused on the beginning of endometrial receptivity, further investigation would be required to confirm whether endometrial receptivity was expanded or simply shifted. Suitable experimental design is discussed in greater detail in Section 6.2.3 and Chapter 8.

Experimental design utilised within this research was the key to demonstrating endometrial receptivity as early as 88 hours pc. Both natural pregnancy, and synchronous and asynchronous uterine ET were explored. This research was the first to incorporate three or more replicates (recipient mice) at each assessed time-point, which minimised the impact of mouse-to-mouse variability. Although no baseline mean implantation rates were established, 88, 90 and 92 hours pc were selected in an effort to capture both the pre-receptive and receptive endometrial phases. In contrast, historical literature, which was at the cutting edge at the time of publication, data was often limited to natural mouse pregnancy, utilised a maximum of one mouse per assessed time-point, and did not report zero baseline implantation rates (Finn & McLaren, 1967; Potts, 1968; McMaster et al., 1993). As a consequence, previous literature could only suggest when endometrial receptivity occurred within pseudo-pregnant mice. Moreover, data was unable to indicate the impact of mouse-to-mouse variability. The experimental design utilised within this research, therefore, held improved accuracy and statistical robustness compared to previous research.

One study, however, demonstrated endometrial receptivity as early as 50 hours pc following the oviductal transfer of E2.5 embryos into R0.5 recipient mice (Ueda, et al., 2003). Their experimental design included IVF and asynchronous oviductal ET was perfectly suitable for their overall research objective, which was to investigate the embryo's ability to influence endometrial receptivity. However, it was difficult to use data to suggest when endometrial receptivity might begin in natural mouse pregnancy or uterine ET. Nonetheless, the findings of Ueda et al. (2003) provided key insights into the embryos ability to influence the period of endometrial receptivity, which will be discussed further in Section 6.2.3.

A greater understanding surrounding the beginning of endometrial receptivity also improves fundamental knowledge and finer control of *in vivo* implantation murine models. For example, this research could benefit researchers who were interested in exploring molecules associated with early adhesion, the second stage of implantation.

By implementing strict controls surrounding the times of coitus, ET and autopsy, described in Section 6.2.2, combined with the knowledge murine endometrium is receptive by 88 hours pc, researchers could target the molecules of interest with increased precision and statistical robustness.

6.2.3 Premature uterine presence of mature embryos induces early endometrial receptivity in mice

The uterine transfer of mature pre-implantation embryos into uteri, which would not normally be exposed to embryos at that time, has been demonstrated within this thesis to induce early endometrial receptivity. This is the first known documentation of early endometrial receptivity subsequent to uterine ET. Only one other study, which performed oviductal ET, reported similar phenomena (Ueda, et al., 2003). However, researchers suggested that the oviductal transport of pre-implantation embryos was responsible for the early induction of endometrial receptivity. In contrast, pre-implantation embryos within this research were transferred directly into uteri, by-passing oviductal transport. Thus, data suggested that the premature uterine presence of mature pre-implantation embryos likely induced early endometrial receptivity and was not dependent on oviductal transport.

Early endometrial receptivity was suggested by the presence of advanced implantation subsequent to the transfer of E3.5(-) or E4.5(-) embryos into R2.5 recipient mice. Two outcomes were indicative of advanced implantation. Firstly, by 88 hours pc (day-3.5 pc), implantation rates had reached their maximum expected mean numbers of implants per recipient mouse (baselines, Table 66). As discussed in Section 6.2.2, such values correlated with the latter stages of endometrial receptivity rather than the beginning, where low mean implantation rates were expected. In contrast, the resulting mean implantation rates within natural mouse pregnancy remained significantly less than their baseline (9.0 implants per mouse) by 92 hours pc (4.2 implants per mouse; p = 0.012; Table 67). Thus, fully saturated mean implantation rates, at a time where much lower levels of implantation were expected, was indicative of more advanced implantation compared to within their naturally pregnant counterparts.

Table 66 Implantation rates on days-3.5 and 17 pc, following the transfer of E3.5(-) and E4.5(-) embryos into R2.5 recipient mice. Implantation rates were expressed as mean numbers of implants per recipient mouse.

		Embryo developmental age at time of ET	
		E3.5(-)	E4.5(-)
Time of recipient assessment (days pc)	3.5*	8.4 (±2.6)	7.8 (±2.1)
	17.0	9.4 (±1.1)	9.8 (±0.5)
P-value		0.399	0.151

*88 hours pc

Table 67 Implantation rates on days-3.5 and 17 pc of natural mouse pregnancy. Implantation rates expressed as mean numbers of implants per mouse.

		Natural mouse pregnancy
Time of recipient assessment (days pc)	3.5*	4.2 (±2.4)
	17.0	9.0 (±0.9)
P-value		0.012[a]

*92 hours pc
a = significantly different p ≤ 0.05

The second outcome that indicated advanced implantation was the intensity and coverage of blue-bands at the time of autopsy. Prior to euthanasia, mice were administered with blue dye. The resulting blue-bands highlighted increased vascularisation surrounding the implantation site, which was associated with adhesion, the second stage of implantation (Psychoyos, 1961, 1973a, 1973b). The intensity and coverage of blue-bands correlated with the extent of vascularisation, which became more complex as implantation progressed. For example, very faint blue-bands that covered less than 50% of the uterine horn circumference (1+) pointed towards less advanced implantation compared to those that were clear and covered 75% or more uterine circumference (3+).

Following the uterine transfer of E3.5(-) or E4.5(-) embryos into R2.5 recipient mice, at least 66% of blue-bands demonstrated a higher intensity and coverage (3+) compared to their R3.5 counterparts and natural mouse pregnancy, where 96% of blue-bands were graded as a 2+ or less. If the beginning of endometrial receptivity remained uninfluenced by the presence of pre-implantation embryos, one would expect comparable advancement of implantation within both pseudo-pregnant and naturally pregnant mice. However, as indicated by blue-band intensity and coverage, and saturated mean implantation rates subsequent to the transfer of mature donor embryos into R2.5 recipient mice, implantation was well advanced in comparison to natural mouse pregnancy. In order for such advanced implantation to be possible, the beginning of endometrial receptivity would have begun well before 88 hours pc. In order to determine how much earlier endometrial receptivity had begun, identification of zero baseline implantation rates, through a series of time-points prior to 88 hours pc, would be necessary. Potential for further experimentation is discussed in Chapter 8.

Wakuda et al. (1999) and Ueda et al. (2003) were also able to demonstrate the pre-implantation embryos ability to influence the timing of endometrial receptivity. Combined with the lack of comparable uterine ET studies, Wakuda et al. (1999) and Ueda et al. (2003) suggested that the oviductal transport of mature pre-implantation embryos was responsible for the early induction of endometrial receptivity. However, this research demonstrated early endometrial receptivity following uterine ET, which bypass the oviducts. Thus, suggested oviductal transport of mature pre-implantation embryos was not crucial to induce early endometrial receptivity. It is important to note that the literature and this research are in agreement with the mature pre-implantation embryo's influence on endometrial receptivity (Wakuda, et al., 1999; Ueda, et al.,

2003). However, the point of difference is in the possible mechanism of action. This research proposed that the premature uterine presence of mature pre-implantation embryos might also influence endometrial receptivity.

Within this research, uterine ET into R2.5 recipient mice was performed no later than 60 hours pc. However, within natural mouse pregnancy only a small number of morula complete oviductal migration by 60 – 72 hours pc (Hogan, et al., 1994). In contrast, R3.5 recipient mice did not receive donor embryos until 80 hours pc, where the uterine presence of embryos was considered normal at this time. Therefore, not only were R2.5 recipient uteri exposed to pre-implantation embryos prematurely, but embryo developmental age was also substantially more advanced than recipient mouse endometrium (Figure 95). It was plausible that such uterine exposure was responsible for the early endometrial receptivity observed within this research.

The premature uterine exposure of mature pre-implantation embryos could have also led to the early endometrial receptivity observed within Ueda et al. (2003). Initially, oviductal migration appeared normal within their study. However, Ueda et al. (2003) did not experimentally compared the time of uteri entry (42 hours post-oviductal transfer) to natural mouse pregnancy (60 – 72 hours pc; Hogan, et al., 1994). It was also difficult to determine how the numbers of hours post-oviductal transfer translated to hours pc. This was due to the unspecified time of ET on day-0.5 pc. The closest indication came from the developmental age of donor embryos at the time of transfer (6 hours post-fertilization), which were said to be synchronous to recipient endometrium. Within Ueda et al. (2003), 6 hours post-fertilization, translated to at least 7 hours pc, 07:00 on day-0.5 pc. Therefore, assuming that pseudo-pregnant recipient mice experienced coitus at approximately the same time as embryo donor mice, the time of ET would have been between 07:00 - 08:00 day-0.5 pc. As a result, approximately 42 hours post-oviductal transfer translated to roughly 50 hours pc (day-2.5 pc). Consequently, both groups of donor embryos entered the uterus ten hours earlier than what was considered normal within the literature (60 – 72 hours pc) and could therefore account for the early endometrial receptivity observed with Ueda's work (Hogan, et al., 1994; Ueda, et al., 2003).
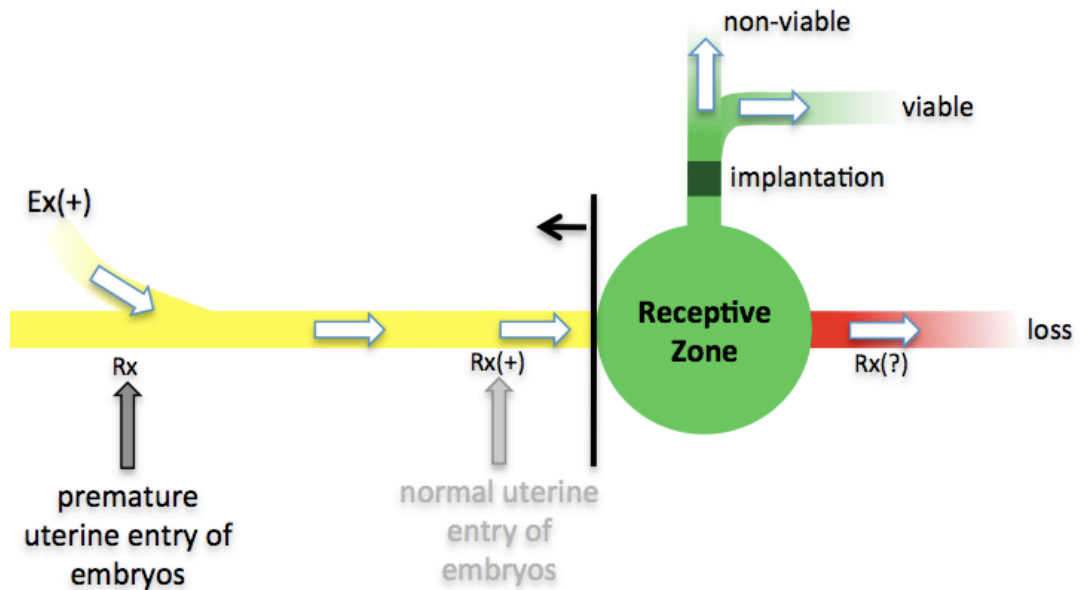
Figure 95 Schematic representation illustrating the impact of premature uteri presence of mature embryos on timing of endometrial receptivity. The transfer of mature embryos (Ex[+]) into recipient uteri prior to normal embryo entry, led to premature uteri exposure of mature embryos. Early endometrial receptivity was then induced.

The premature uterine presence of mature pre-implantation embryos could potentially influence the timing of endometrial receptivity through a number of mechanisms. Within R2.5 recipient mice the time available for E3.5(-) and E4.5(-) embryos to become implantation-competent and implant exceeded what was required. Once that competency was reached, donor embryos could communicate their readiness to the less advanced endometrium. Possible mechanisms include: 1) direct trophectoderm-endometrial epithelial cell contact; 2) endometrial ligand interaction with the embryonic molecular cell-surface profile; or 3) paracrine release of embryonic cytokines, for example interleukin-1α and β, which have been shown to lead to endometrial transformation (Aplin, 1997, 2000, 2006; Armant, 2005; Makker & Singh, 2006). Such increased cross-talk between donor embryos and recipient endometrium may also hold the potential to verify the ability of the Suboptimal Model (Section 6.1.4), which will be expanded upon in Chapter 8.

Another area worthy of exploration is whether early endometrial receptivity was a product of expansion or a shift of that period. Although this study could not confirm either theory, Ueda et al. (2003) provided some insight. Within their study, R0.5 recipient mice received both E0.5 and E2.5 embryos (separate uterine horns). While the baselines of each group of embryos were comparable (72%; 79%; p > 0.05), the

earliest detection of implants differed by more than 24 hours (Ueda, et al., 2003). Although not statistically significant, 5% of E2.5 embryos had begun to implant as early as 50 hours pc. By 74 hours pc (day-3.5 pc), 63% of E2.5 embryos, which would have become implantation-competent 24 hours earlier, had already implanted. In contrast, E0.5 embryos did not result in implants (59%) until 98 hours pc (day-4.5 pc) (Ueda, et al., 2003). Recipient mouse endometrium had therefore allowed implantation for at least 24 hours, potentially 12 hours longer than previously indicated within the literature (Doyle, et al. 1963; Ma, et al., 2003; Paria, et al., 1993; Psychoyos, 1973a, 1973b; Yoshinaga, 1988). If endometrial receptivity had simply shifted, the endometrium would have become refractive by approximately 74 hours pc (R3.5), preventing E0.5 embryos from implanting. Therefore, Ueda et al. (2003) suggested that the period of endometrial receptivity had expanded within this research.

An expanded period of endometrial receptivity could enable increased implantation opportunity. Clinical interventions, such as fresh human-IVF, could benefit from such increased opportunity. In response to superovulatory-drugs, endometrial development is advanced for its real-time age (Ertzeid & Storeng, 2001; Ertzeid, Storeng, & Lyberg, 1993; Oehninger, 2008; Sibug, et al., 2007). As a result, the time available for transferred human embryos to become implantation-competent and implant, is reduced compared to non-superovulated cycles. Whether the expansion was a product of early endometrial receptivity or an extension of its final stages, would determine its translation to live birth outcomes. Only an extended period of receptivity would increase implantation opportunity for transferred human embryos. In contrast, an early induction of endometrial receptivity would only benefit those embryos that were close to implantation-competency at the time of transfer. It would also be important, however, to ensure increased implantation opportunity did not allow chromosomally abnormal embryos from implanting, which could lead to future miscarriage or elective abortion. Neither outcome is preferable for patients. Chromosomal testing prior to transfer of IVF embryos could minimise this risk (Scott R. T. Jr., Ferry, Su, Tao, Scott, K., & Treff, 2012).

Further investigation was required to determine whether the premature uterine presence of mature embryos could also extend endometrial receptivity. An extension of endometrial receptivity could be indicated by the presence of implants within recipient mice whose endometrium was considered refractive at the time of ET. However, such experimental design did not allow for the premature uterine presence of

mature embryos. Alternatively, embryo transfer prior to normal embryo uterine entry (60 – 72 hours pc, day-3.0 pc), combined with scanning electron microscopy detection of pinopodes, indicative of endometrial receptivity, may be a viable option (Achache & Revel, 2006; Makker & Singh, 2006; Murphy, 2000a, 2000b, 2004). Following ET, recipient mice would be assessed for presence of pinopodes during and after endometrial receptivity. Detection of pinopodes within recipient mice, whose endometrium was no longer thought to be receptive at the time of assessment, would indicate extended receptivity. If extended to human IVF scenarios it would be important to include hormonal manipulation to prevent premature luteinisation and menstrual feedback (Depalo, et al., 2012). Although such experimentation exceed the scope of this thesis, the possibilities that they bring for further research and potential commercial benefits were exciting.

7   Concluding remarks

7.1 Research rationale

Implantation is crucial for successful pregnancy. However, due to the ethical and legal constraints of human research, little knowledge surrounds embryo implantation (Hannan, et al., 2010; Lee & DeMayo, 2004; Rogers, 1995). As a consequence, scientists have utilised numerous alternative tools to explore embryo implantation. One such tool is *in vivo* implantation murine modelling (Carson, et al., 2000; Makker & Singh, 2006). Although these models have offered substantial insight, none are recognised as standard. This was due to increased experimental variability, within and between research groups, which made critical analysis and comparison difficult.

An extensive literature review revealed four potential contributors towards the lack of standardization. These included: 1) suitable statistical analysis was often not reported, making mouse-to-mouse consistency difficult to ascertain within individual studies; 2) inconsistent implantation rates were apparent between different research groups, which used comparable models; 3) the beginning of endometrial receptivity, critical for successful implantation, was not clearly defined; and 4) the impact of altered embryo-endometrial developmental synchrony, in terms of hours not days, on the timing of endometrial receptivity and resulting implantation rates had not yet been thoroughly explored. It was proposed that model standardization would strengthen experimental data, enable greater comparison between research groups and aid development of effective research platforms that were useful to explore implantation and preliminary studies surrounding IVF-intervention potential. Therefore, the overall research objective was to gain insight into improved standardization of *in vivo* implantation murine models that were suitable for such a purpose.

7.2 Contribution towards research objective

The findings of this research provided insight into improved *in vivo* implantation murine modelling standardization, which contribute towards overall research objective. The ET outcomes of a number of optimal and suboptimal *in vivo* implantation murine models on day-17 pc, and the ability of the most suitable optimal and suboptimal models to detect consequences of manipulated embryos, a potential IVF-intervention, facilitated this achievement. Although, examination provided fundamental knowledge surrounding

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murine implantation rates, data also revealed inconsistencies within models that exhibited low levels of implantation. The literature shed little light on mouse-to-mouse variability. In fact, gaps in knowledge surrounding the beginning of endometrial receptivity and how altered embryo-endometrial developmental synchrony influenced its timing, were identified. For this reason, the beginning of endometrial receptivity within natural mouse pregnancy and pseudo-pregnancy, using synchronous and asynchronous ET as a vehicle, were examined. Three points were revealed:

1. Implantation rate consistency, within models that exhibited low levels of implantation, was influenced by the distinct moments of coitus, ET and autopsy, in terms of hours. This contrasted the literature, which often focused on days.

2. Naturally pregnant mouse endometrium was receptive by 88 hours pc, four hours earlier than previously reported, which could translate to a 33% increase in the total length of normal endometrial receptivity.

3. The premature presence of mature pre-implantation embryos following uterine transfer into recipient mice, whose endometrium would not normally be exposed to embryos at that time, identified an additional mechanism of action leading to early endometrial receptivity.

Sections 7.3 and 7.4 summarise the above findings.

7.3 Deficiencies of existing optimal and suboptimal in vivo implantation murine models

The focus of this research was to gain an understanding of improved standardization of *in vivo* implantation murine models, suitable to explore both implantation and the potential of embryo manipulation, an IVF-intervention. Part of that process was to establish foundational implantation knowledge surrounding optimal and suboptimal implantation. A comparison of a selection of existing *in vivo* implantation murine models with natural mouse pregnancy on day-17 pc formed initial experimentation. Investigation of the most suitable optimal and suboptimal models, for their ability to detect consequences of manipulated embryos followed. Existing *in vivo* implantation murine models included: ET into SO-recipient mice (advanced endometrium), progesterone antagonist administration following ET (delayed endometrium) and synchronous and asynchronous ET (a deliberate shift in embryo-endometrial developmental age at the time of transfer).

This research was able to confirm the influence of two variables on mean murine implantation rates. While previous literature clearly linked increased implantation rates with a reduced length of time required for embryo implantation-competency, no clear link could be established with the length of time available for that competency. In contrast, this research indicated that both the time required and available for embryo implantation-competency influenced mean implantation rates. When the time required by embryos to reach implantation-competency decreased, mean implantation rates increased. Furthermore, an increase in the time available for embryo implantation-competency also saw an increase in mean implantation rates. Therefore, this research highlighted the importance of considering both of these times when selecting the level of implantation suitable to explore different aspects of embryo manipulation potential.

The most suitable optimal and suboptimal models offered the opportunity to assess embryo manipulation as a potential IVF-intervention. However, before either could be utilised, it was important to verify whether consequences of manipulated embryos could be detected. Findings verified the Optimal Model's ability to detect negative consequences of embryo manipulation. For this reason, the Optimal model could be used to assess the safety of an embryo manipulation. In contrast, free and inserted-HA were unsuitable to confirm the ability of the Suboptimal Model to detect positive consequences of manipulated embryos. Further research would be required to select, generate, and trial additional molecule suitable for this purpose. A potential candidate may be embryo manipulation that mimics the surface expression profile of implantation-competent embryos. Such manipulation may imitate the induction of early endometrial receptivity observed within Chapter 5. As a consequence, implantation opportunity and resulting implantation rates may increase. If an ability to detect positive consequences could be confirmed, the Suboptimal Model could prove useful to assess the potential embryo manipulation held to improve suboptimal implantation rates.

While *in vivo* implantation murine models that achieved high levels of implantation were consistent, models that exhibited low levels of implantation, including the Suboptimal Model, demonstrated significantly inconsistencies, where individual implantation rates varied up to ± 4 implants of their mean. As a consequence, individual implantation rates within models, whose mean was considered suboptimal, could be either optimal or suboptimal. It is possible that the uterine environment and thus implantation

opportunity within models was incomparable mouse-to-mouse. Therefore, without improved implantation rate consistency, there was a concern that any subtle, but significant data associated with manipulated embryos could be masked.

As stated in Section 6.1, extensive literature reviews revealed little insight towards implantation rate consistency mouse-to-mouse. In contrast, gaps in knowledge were detected, which included the beginning of endometrial receptivity and how altered embryo-endometrial developmental synchrony influenced that time. Consequently, this research investigated the beginning of endometrial receptivity within natural mouse pregnancy, and for the first time within uterine ET, using synchronous and asynchronous ET as a vehicle. Subsequent findings and their significance are summarised in Section 6.4.

7.4 Research field extended

7.4.1 Requirements identified for improved *in vivo* implantation murine model standardization

For the first time, the influence of the distinct moments of coitus, ET and autopsy on implantation rate consistency has been demonstrated in terms of hours not days. Previous literature, which provided crucial foundational scientific knowledge, focused on days. Within *in vivo* implantation murine models that exhibited high levels of implantation, the moments of coitus, ET and autopsy exerted little impact on implantation rate consistency. In contrast, the moments of coitus, ET and autopsy influenced models that achieved low levels of implantation. These distinct moments determined the length of time available for embryo implantation-competency and subsequent implantation. Thus any variation in the distinct moments of coitus and ET, in context of the time of autopsy, resulted in an unpredictable length of time available for embryo-implantation competency. As a result, implantation opportunity within *in vivo* implantation murine models that exhibited low levels of implantation could differ from mouse-to-mouse, resulting in inconsistent implantation rates within those models.

Investigation of the beginning of endometrial receptivity also suggested how tighter control of coitus, ET and autopsy, could improve implantation rate consistency within models that exhibited low levels of implantation. Following the transfer of E4.5 embryos into R3.5 recipient mice, individual implantation rates at 88, 90 or 92 hours pc

(day-3.5 pc) varied by up to only ± 2 implants of their mean values verses ± 4 implants on day-17 pc. Although numbers of replicates would need to be increased to confirm this observation, data did suggest the potential benefit in introducing tighter controls surrounding coitus, ET and autopsy. Combined with the need to carefully consider the time required and available for embryo implantation-competency, as highlighted in Section 6.3, implementing such controls, may help improve standardisation of *in vivo* implantation murine models.

7.4.2 Improved fundamental knowledge surrounding murine endometrial receptivity

This research has gone further than previous literature, which did not report endometrial receptivity within natural mouse pregnancy prior to 92 hours pc. In contrast, this research demonstrated murine endometrial receptivity as early as 88 hours pc within both natural pregnancy, and synchronous and asynchronous uterine ET. Unexpectedly, no zero baseline mean implantation rates were exhibited. In addition, following transfer of mature embryos into R2.5 recipient mice, maximum mean numbers of implants were achieved by 88 hours pc. Such saturation was indicative of the later stages of endometrial receptivity rather than the earlier stages, where lower levels of implantation would be expected. Data, therefore, extrapolated that murine endometrial receptivity could begin prior to the earliest assessed time-point (88 hours pc).

A four-hour difference was considered significant given that murine endometrial receptivity is thought to be at least 12 hours, but less than 24 hours in length. Such a difference could translate to a potential 33% increase in known implantation opportunity. While the literature presented crucial foundational knowledge, trials were often limited to one mouse per assessed time-point (one replicate). In contrast, this research utilised three to five mice per time-point (three – five replicates). While the numbers of mice were not substantially larger, resulting data did have improved statistical strength from previous research. Moreover, this is the first known study to report endometrial receptivity within both naturally pregnant and pseudo-pregnant recipient mice.

The early induction of endometrial receptivity was observed following the transfer of mature embryos into uteri, whose endometrium would not normally be exposed to embryos at that time. Prior to this research, the pre-implantation embryo's ability to

induce early endometrial receptivity had not been reported following uterine ET. Only one other study, which performed oviductal ET, demonstrated a similar phenomenon (Ueda, et al., 2003). Due to the lack of uterine ET evidence at the time, researchers rightly suggested that the oviductal transport of pre-implantation embryos was responsible for the early induction of endometrial receptivity. In contrast, mature pre-implantation embryos were transferred directly into uteri within this research, by-passing their oviductal transport. For this reason, findings elucidated another possible mechanism of action, where the premature uterine presence of mature pre-implantation embryos could also induce early endometrial receptivity.

Improved knowledge surrounding murine endometrial receptivity and the influence of pre-implantation embryos on it's timing benefits the greater scientific community. Dissemination of this research would enable researchers to target the beginning of endometrial receptivity with greater accuracy and efficiency. In addition, research surrounding human embryo co-culture with 3D *in vitro* human endometrial constructs or with mouse uterine explants, could indicate whether human embryos also held an ability to influence endometrial receptivity. Resulting data could suggest whether exploiting human embryos in this way could influence implantation opportunity as an IVF-intervention.

8 Suggestions for future research

Due to the nature of animal experimentation, this research was subject to ethical approval, which was given for a set period of time. This research had been part of an ongoing larger project, which by the end of this work had already renewed its ethical approval to the maximum renewals allowed (three times). The expiration of this approval and financial constraints prevented further investigation surrounding the refinement of the Suboptimal Model and the premature uterine presence of pre-implantation embryos on the beginning of endometrial receptivity. Seven potential areas of interest have been identified.

The first potential area for future research surrounded improvements of current experimental design. The simplest of improvements included increasing the numbers of replicates (recipient mice), and restricting coitus to a smaller window of opportunity. Both would have contributed to a clearer picture surrounding mouse-to-mouse variability. In particular, increasing the numbers of replicates when exploring the beginning of endometrial receptivity would have increased statistical strength of findings. In addition, the introduction of fetal sex determination, by chromatin analysis. The differences in male:female ratios could have explained subtle differences seen in fetal/placental weights. In context of baseline investigations, duplicate sets of recipient mice, which were allowed to give birth, could have provided an insight into long-term post-natal health. However, this would have substantially increased experimental numbers of mice, which was financial and colony housing constraints of this research.

Three other improvements could have added to current experimental design. Gestational weight and nutrition intake of recipient mice would have provided another aspect to model consequences. In addition, introduction of sham controls would have also strengthened data resulting from ET into SO recipient mice, and progesterone antagonist administration following ET. Their lack of inclusion reflected the early stage experiments were completed in along the research journey. In addition, any potential detriment effects of the ET procedure itself could have been claried by a sham transfer into naturally pregnant mice. The introduction of IVF or intracytoplasmic sperm injection could have also eliminated any natural variation in donor embryo developmental age. Such techniques would standardize the length of time required by donor embryos to reach implantation-competency.

The second area for potential future research was an expansion of model scenarios. These included additional synchronous and asynchronous uterine ET scenarios. For example, the asynchronous transfer of E3.5 or E4.5 embryos into R1.5 recipient mice. This asynchronous uterine ET scenario could help explore consequences of early ET on implantation rates. A further scenario would be the synchronous transfer of *in vivo* cultured E3.5 embryos into R3.5 recipient mice, which could provide an additional comparison to natural mouse pregnancy. Moreover, data could offer an insight into *in vitro* culture effects within this research.

If time and financial expense were not a limiting factor, it would have also been interesting to explore a number of other mouse strains. For example, the ICR or CD-1 strains utilised by McMaster et al. (1993) and Ueda et al., (2003). Comparison between strains could identify whether the beginning of endometrial receptivity is strain dependent. In addition, knock out or knock-down mouse strains that targeted selectins involved in initial apposition and fetal-maternal cross-talk could be explored. Donor embryos null of the selectin of interest may have defective or zero implantation capability. These embryos could be modified to express the missing selectin and then transferred into normal pseudo-pregnant recipient mice to see if implantation levels could be restored. Exploring the impact of recipient age on implantation rates would also have been an interesting area of research. Within the current thesis most recipient mice were between 60 and 80 days old pb, with a maximum age of 100 days pb. Inclusion of a second group of recipient mice, who were older than 100 days pb, would have offered a useful comparison. Typically, older recipient mice have reduced ovulation and general fertility, which would be similar to older women seeking ART intervention.

Refinement of the Suboptimal Model offered the third potential area for future research. As identified within Section 4.4, the Suboptimal Model met the second selection criterion only in part. While the model's baseline (day-17 pc) met the preferred mean of 6 implants per recipient mouse, variability of ± 4 implants exceed acceptable limits (± 1 implant). Investigation of the beginning of endometrial receptivity revealed that the strict control of coitus, ET and autopsy improved implantation rate consistency (± 2 implants). It is plausible, therefore, that combining such control with a time-point located towards the end of endometrial receptivity, may enable the Suboptimal Model to meet the second criterion in whole. Validation of such improvements would bring the standardization of the Suboptimal Model one step closer.

Establishing the beginning of endometrial receptivity within natural mouse pregnancy and pseudo-pregnancy was the fourth area of interest. Of particular interest was the asynchronous transfer of E3.5(-) or E4.5(-) embryos into R2.5 recipient mice. Identification of zero baseline implantation rates, through a selection of time-points located prior to 88 hours pc, could achieve the desired outcome. Due to the advanced nature of implantation following the asynchronous transfer of mature pre-implantation embryos into R2.5 recipient mice, it was likely that assessed time-points would need to extend beyond those of natural mouse pregnancy and remaining synchronous and asynchronous ET scenarios. Identification of the beginning of endometrial receptivity within a number of different scenarios, would improve the accuracy at which researchers could target the early stages of implantation.

The fifth area of interest surrounded the premature uterine presence of pre-implantation embryos and their influence on endometrial receptivity. It would be of interest to identify whether early endometrial receptivity was dependent on both the premature uterine presence of embryos and their developmental age. Experimentation could include the transfer of E1.5(-), 2.5(-), 3.5(-) and 4.5(-) embryos into R2.5 recipient mice, whose endometrium would not normally be exposed to embryos at that time. If zero baselines were identified at the same point in time for each group of embryos, data would suggest that early endometrial receptivity, following the premature uterine presence of pre-implantation embryos, would not be dependent on embryonic developmental age.

It would also be of interest to determine whether the early initiation of endometrial receptivity, seen following the premature uterine presence of mature pre-implantation embryos, was a product of an expansion or a shift in that endometrial phase. Exploration of this area offered the sixth area of potential future research. Moreover, exploring the pre-implantation embryo's ability to extend endometrial receptivity may hold the potential to improve implantation opportunity within IVF-cycles. Embryo transfer prior to 60 hours pc (normal completion of oviductal transport), combined with the scanning electron microscopy detection of pinopodes, indicative of endometrial receptivity, could reveal how mature pre-implantation embryos influenced the receptive phase (Achache & Revel, 2006; Makker & Singh, 2006; Murphy, 2000a, 2000b, 2004). Detection of pinopodes at time where the endometrium considered pre-receptive or no longer receptive, would indicate whether endometrial receptivity had expanded, shifted or extended.

Gene arrays or the study of metabolomics could also suggest whether the endometrial receptivity had expanded or shifted. The detection of key genes or metabolites could indicate endometrial receptivity status at any one time. For example, at the time of implantation, and thus endometrial receptivity, LIF expression is high, but MUC-1 expression is low (Table A1 and A2). If a similar pattern of expression was detected outside the normal period of endometrial receptivity, data could indicate an expansion or shift of that receptivity.

The seventh and final potential area for future research identified would be to confirm the ability of the Suboptimal Model to detect positive consequences of manipulated embryos. Understanding the mechanism of action surrounding early endometrial receptivity may provide some opportunities. One potential mechanism included endometrial ligand interaction with the embryonic molecular cell-surface profile. Embryo manipulation could exploit such embryonic molecules, so that the cell-surface expression profile of implantation-competent embryos was mimicked. In response, the receptive endometrial phase could be altered so that implantation opportunity was increased. However, this would be dependent on whether mature pre-implantation embryos expanded, shifted or extended endometrial receptivity. In addition, it would be important to consider the potential negative impact of rescuing embryos that would not normally survive. In particular, if introduced commercially, a technique, such as chromosomal testing, would need to be utilised alongside embryo manipulation to minimise the risk of future miscarriages or elective abortions. Nonetheless, such manipulated embryos may be able to confirm the Suboptimal Model's ability to detect positive consequences of that intervention.

Extending current research could further establish the standardization requirements identified within this research. Implementation of these requirements would facilitate strengthened experimental data within *in vivo* implantation murine models, and also provide a common scientific framework, enabling greater comparison between research groups. Moreover, introduction of the identified requirements to the wider academic community could enable exploration of fundamental implantation knowledge with greater depth and precision. For example, investigation of adhesion molecules associated with the second stage of implantation. In addition, standardised models could help improve assessment of clinical IVF-intervention potential, which hold profitable value.

9   Appendix – Supplementary literature

Table A1 Key adhesion molecules involved in embryo adhesion, the second stage of implantation. Information sourced from Aplin, 1997, 2006; Aplin & Kimber, 2004; Armant, 2005; Armant, et al., 2000; Carson, et al., 2000; Dey, et al., 2004; Fukuda & Sugihara, 2008; Hoozemans, Schats, Lambalk, Homburg, & Hompes, 2004; Singh, et al., 2011; Wang & Dey, 2006.

Molecule type	Example	Function / Type of binding	Expression time and location	Bioactive
Basigin (CD147)	n/a	Immunoglobulin superfamily member	High endometrial epithelium expression day-3.5 pc in response to embryo	
Cadherins	Cad-11, E-cadherin	Important for anchorage. Homotypic adhesion, mediated by calcium	Embryonic trophectoderm and endometrial epithelium.	Calcium
Cabrohydrates	Le^Y	Potential adhesion involvement through carbohydrate-to-carbohydrate.	Embryonic trophectoderm	Htype1
CD44	n/a	Cell surface glycoprotein that serve basic protein interaction	Expressed up to and including blastocyst stage of pre-implantation embryo	Integrins, hyaluronan, osteopontin, chrondroitin sulfate
Galectins	Galectin-1	Carbohydrate-lectin binding	Trophectoderm of expanded blastocyst	β-galactosides
Heparin Sulfate Proteoglycans (HSPG)	Perlecan	HSPG-protein interaction. Binds to HS-interacting protein, which is expressed in glandular and luminal epithelium.	Implantation-competent trophectoderm, and endometrial epithelium (pinopodes/uterodomes). Highly regulated by steroidal hormones.	ECM proteins, growth factors and cytokines
H-type-1	n/a	Carbohydrate-carbohydrate binding	Endometrial epithelium at time of implantation, associated with MUC-1.	Le^Y expressed on trophectoderm.
Hyaluronan	n/a	Many roles outside of implantation. Major ECM component. May serve cell-to-cell, and cell-to-ECM adhesion during implantation.	Expressed in endomentrial stroma all of human menstrual cycle, but highest during endometrial receptivity.	CD44
Integrins	αvβ3, αvβ5	Bi-functional extracellular bridging ligands (transmembrane glycoproteins)	Endometrial epithelium, and embryo in response to HB-EGF	Fibronectin, vitronectin, osteopontin, thrombospondin, laminin
Glycoproteins	MUC-1	Down regulation allows ECM clearance and implantation.	ECM – low expression at time of murine implantation. High expression at time of human implantation, except for implantation site.	L-selectin (humans only), fibronectin, vitronectin, laminin, collagen type IV
Osteopontin	n/a	Linking protein	Secreted by glandular epithelium in response to high levels of progesterone	Integrins, CD44
Selectins	L-selectin	Traditionally involved in leukocyte capture in bloodstream. Maybe involved in gradual tethering of embryos	Embryonic trophectoderm and endometrial epithelium. Unique to human endometrium.	MUC-1, L-selectin receptors (trophectoderm)

| Trophinin-tastin-bystin complex | n/a | Homotypic adhesion | Embryonic trophectoderm and endometrial epithelium (pinopodes/uterodomes). Specific to human implantation site. Mouse expression at time of implantation. | |

Table A2 Key growth factors and cytokines involved in embryo adhesion, the second stage of implantation. Note some have multiple roles from uterine preparation through to decidualization. Information from Dey et al. (2004), Dimitriadis et al. (2005), Singh et al. (2011) and Wang & Dey (2006.)

Molecule type	Example	Function / Type of binding	Expression time and location	Bioactive
Calcitonin	n/a	Stimulates embryo to express fibronectin receptor α5β1. May regulate implantation by autocrine/paracrine action.	Expressed in endometrial epithelium around the time of implantation. Regulated by progesterone.	-
Colony-stimulating factor-1	n/a	Cytokine. May maintain normal uterine macrophage population during early pregnancy and stimulate macrophages to secrete cytokines.	Null-mice exhibit decreased implantation rates and fetal viability.	-
COX-2 derived prostaglandins	Prostacyclin (PGI$_2$)	Important in vascular responses, cellular proliferation and differentiation.	Expressed in endometrial epithelium at the time of implantation. COX-2 deficient mice have defective ovulation, fertilization and implantation.	Peroxisome proliferator-activated receptor-δ. Important for implantation and decasualization.
Epidermal growth factors	Heparin binding-EGF, amphiregulin, transforming growth factor-β	TGF-β regulates proliferation and differentiation of ECM, implantation via stimulating fibronectin/vascular endothelial growth factor/LIF expression, and placental development and function. HB-EGF involved in implantation and regulates LIF expression.	Endometrial epithelium – membrane bound. HB-EGF expression indicator of receptive endometrium.	ErbB4 trophectoderm receptor

Homeobox proteins	Hox10a, Hox11a	Uterine preparation, implantation and decidualization. Hox11a more crucial, null mice do not express LIF.	Uterine stroma during receptive phase, persisting during post-implantation decidualization.	-
Interleukins	IL-6, IL-11	Cytokines. Both important for uterine preparation. IL-6 role in trophoblast growth and placental development. IL-6 has some functional redundancy with IL-11 and LIF in context of uterine receptivity.	Glandular and luminal epithelium.	Binding with α-receptor dimerizes gp130 protein, which activates JAK/STAT signal transduction pathway.
Leukaemia inhibiting factor (LIF)	n/a	Cytokine. Important for uterine preparation and later in attachment reaction.	Highly expressed in endometrial glands at time of implantation. Also expressed in stromal cells surrounding embryo during attachment. Not directly influenced by steroidal hormones, but rather growth hormones and cytokines. Defective implantation in LIF-null mice, which lack HB-EGF expression at implantation site just prior to attachment.	Binding with α-receptor dimerizes gp130 protein, which activates JAK/STAT signal transduction pathway.
Tumour necrosis factor-α		Cytokine	NB: null mice are still fertile – molecular redundancy.	

Table A3 Embryo transfer variables and resulting data associated with individual studies that utilised synchronous and asynchronous ET and ET into SO recipient mice to generate *in vivo* implantation murine models. Areas of grey and white group key information. First grey section corresponds to recipient mice, second white section corresponds to donor embryos, and second grey section corresponds to ET. Key: SO, superovulated; N, naturally ovulated; U, urinary.

Researcher	Implantation rate (%)	Viability rate (%)	Recipient mouse strain	Recipient number	Time of assessment (days pc)	Embryo donor mouse age	Naturally or superovulated	Urinary or recombinant gonadotropin	Day of embryo retrieval (pc)	Embryo culture status	Number of embryos transferred per recipient	Number of embryos transferred per uterine horn	Unilateral or dual horn transfer
Model: Synchronous ET E2.5/R2.5													
McLaren and Michie 1955	11	-	C3H x C57BL	9	16.5	prepub	SO	U	3.5	*in vivo*	mean of 10	-	Unilateral
Lane and Gardner 1994	39	17	C57BL/6 x CBA/Ca	-	15	prepub	SO	U	0.5	*in vitro*	-	-	-
Model: Asynchronous ET, E3.5/R0.5													
Wakuda et al. 1999	0	-	ICR	20	11	adult	SO	U	3.5	*in vivo*	6	3	Dual horn
Model: Asynchronous ET, E3.5/R1.5													
Doyle et al. 1963	0	0	BALB/c x C57BL	11	> 18	adult	N	-	3.5	*in vivo*	-	-	Dual horn
Nakayama et al. 1995	-	2	ICR	46	10	prepub	SO	U	3.5	*in vivo*	2	-	-
Wakuda et al. 1999	0	-	ICR	20	11	adult	SO	U	3.5	*in vivo*	6	3	Dual horn

Goto et al.1993	2	-	ICR	12	11.5	adult	SO	U	3.5	*in vivo*	5 - 7		Unilateral

Model: Asynchronous ET, E3.5/R2.5

McLaren and Michie 1955	22	-	C3H x C57BL	15	16.5	prepub	SO	U	3.5	*in vivo*	mean of 9	-	Unilateral
McLaren and Michie 1955	23	-	C3H x C57BL	16	16.5	prepub	SO	U	3.5	*in vivo*	mean of 9	-	Unilateral
Lim et al. 2006	26	-	ICR	13	4.5	adult	SO	U	2.5	*in vitro*	20	10	Dual horn
Wakuda et al. 1999	52	-	ICR	20	11	adult	SO	U	3.5	*in vivo*	6	3	Dual horn
Lane and Gardner 1994	52	36	C57BL/6 x CBA/Ca	-	15	prepub	SO	U	0.5	*in vitro*	-	-	-
Mullen and Carter 1973	65	49	C57BL/10G nDg	12	16-17	-	N	-	2.5	*in vitro*	8 – 14	-	Dual horn
Mullen and Carter 1973	72	65	(C57BL/10G nDg x CBA/J) F$_1$	12	16-17	-	N	-	2.5	*in vitro*	8 – 14	-	Dual horn
Doyle et al. 1963	-	43	BALB/C x C57BL	11	> 18	adult	N	-	3.5	*in vivo*	-	-	Dual horn
Nakayama et al. 1995	-	64	ICR	46	10	prepub	SO	U	3.5	*in vivo*	2	-	-
Weibold and Anderson 1986	-	66	-	-	> 18	-	-	-	-	*in vitro*	-	-	-

Model: Synchronous ET, E3.5/R3.5

McLaren and Michie 1955	11	-	C3H x C57BL	15	17.5	prepub	SO	U	3.5	*in vivo*	mean of 7	-	Unilateral
Paria et al. 1993	47	-	CD-1	5	4.5	adult	N	-	3.5	*in vivo*	12	5 - 7	Dual horn

Reference												
Fossum et al. 1989	50	-	C6B3F1	4	8.5	adult	N	-	3.5	*in vitro*	5	5	Unilateral
Paria et al. 1993	51	-	CD-1	5	4.5	adult	N	-	3.5	*in vivo*	12	5 - 7	Dual horn
Wakuda et al. 1999	51	-	ICR	20	11	adult	SO	U	1.5	*in vivo*	6	3	Dual horn
Paria et al. 1993	52	-	CD-1	5	4.5	adult	N	-	3.5	*in vivo*	12	5 - 7	Dual horn
Song et al. 2007	52	-	-	5	5.5	adult	N	-	3.5	*in vivo*	12	-	Dual horn
Goto et al. 1993	58	-	ICR	16	13.5	adult	SO	U	3.5	*in vivo*	max 7	-	Unilateral
Rulicke et al. 2006	79	-	Zbz:FM	2	18	adult	SO	U	1.5	*in vitro*	7	7	Unilateral
Doyle et al. 1963	-	50	BALB/C x C57BL	11	> 18	adult	N	-	3.5	*in vivo*	-	-	Dual horn
Nakayama et al. 1996	-	45	ICR	49	10	prepub	SO	U	3.5	*in vivo*	2	-	-
Wiebold and Anderson 1986	-	42	-	-	> 18	-	-	-	-	*in vitro*	-	-	-

Model: Asynchronous ET, E3.5/R4.5

Reference												
Wakuda et al. 1999	3	-	ICR	20	11	adult	SO	U	3.5	*in vivo*	6	3	Dual horn
Rulicke et al. 2006	36	-	Zbz:FM	3	18	adult	SO	U	1.5	*in vitro*	mean of 8	mean of 8	Unilateral
Song et al. 2007	48	-	-	7	6.5	adult	N	-	3.5	*in vivo*	12	-	Dual horn

Model: Asynchronous ET, E3.5/R5.5

Reference													
Rulicke et al. 2006	0	-	Zbz:FM	3	18	adult	SO	U	1.5	*in vitro*	mean of 7	mean of 7	Unilateral
Wakuda et al. 1999	0	-	ICR	20	11	adult	SO	U	3.5	*in vivo*	6	3	Dual horn

Model: Asynchronous ET, E4.5/R3.5

Reference													
Paria et al. 1993	0	-	CD-1	5	5.5	adult	N	-	3.5	*in vivo*	12	5 - 7	Dual horn
Gardner et al. 1999	53	36	C57BL/6 x CBA/Ca	≥ 4	15	prepub	SO	U	0.5	*in vitro*	10	5	Dual horn
Kelley et al. 2006	54	21	CBA/C57 F1	16	15	prepub	SO	U	0.5	*in vitro*	12	6	Dual horn
Gardner et al. 1999	56	44	C57BL/6 x CBA/Ca	≥ 4	15	prepub	SO	U	0.5	*in vitro*	10	5	Dual horn
Cheng et al. 2004	70	-	ICR	16	5.5	adult	SO	U	0.5	*in vitro*	-	-	-
Lane and Gardner 1994	78	50	C57BL/6 x CBA/Ca	-	15	prepub	SO	U	0.5	*in vitro*	-	-	-
Doyle et al. 1963	-	21	BALB/C x C57BL	11	> 18	adult	N	-	3.5	*in vivo*	-	-	Dual horn

Model: ET into SO recipient mice, E3.5/R2.5

Reference													
Ertzeid and Storeng 2001	7	11	C57	-	17	adult	N	U	1.5	*in vitro*	2 - 6	2 - 6	Dual horn
Ertzeid and Storeng 2001	10	31	C57	-	17	adult	SO	U	1.5	*in vitro*	2 - 6	2 - 6	Dual horn
Van der Auwera et al. 2001	62	32	CBA/C57 F1	21	13	prepub	SO	U	1.0	*in vitro*	5	5	Dual horn

Model: ET into SO recipient mice, E3.5/R3.5

Fossum et al. 1989	8	0	C6B3F1	8	8.5	adult	SO	U	3.5	*in vitro*	5	5	Unilateral

Model: ET into SO recipient mice, E4.5/R3.5

|												
Kelley et al. 2006	5	2	CBA/C57 F1	19	15	prepub	SO	U	0.5	*in vitro*	12	6	Dual horn

Table 68 Consequences of high dosage progesterone antagonist administration into the Optimal Model one-day post-transfer. Progesterone antagonist (RU486) subcutaneously administered into eight recipient mice, at 3.0 x10^{-5} g / 10 g of body weight, one-day post-transfer. Mean numbers of implants, viable fetuses, non-viable fetuses and losses, and the mean fetal and placental weights were assessed on day-17 pc. Only one recipient mouse resulted in implants. Mean numbers of implants, viable and non-viable fetuses, mean fetal weight and fetal:placental weight ratio were significantly reduced (p < 0.001). In contrast, mean numbers of losses, and the mean placental weight were significantly increased (p < 0.001).

Outcome	Unit	Progesterone antagonist administration one-day post-ET		Optimal Model		P-value of means
		Mean per mouse (±SD)	Total	Mean per mouse (±SD)	Total	
Implants viable + non-viable fetuses	n	0.9 (±2.5)	7	9.4 (±1.1)	122	< 0.001[a]
Viable fetuses	n	0.3 (±0.7)	2	6.9 (±2.1)	90	< 0.001[a]
Non-viable fetuses	n	0.6 (±1.8)	5	2.5 (±1.8)	32	< 0.001[a]
Losses	n	9.1 (±2.5)	73	0.5 (±1.1)	6	< 0.001[a]
Fetal weight	g	0.9 (±0.04)	-	1.0 (±0.2)	-	< 0.001[a]
Placental weight	g	0.15 (±0.03)	-	0.1 (±0.02)	-	< 0.001[a]
Fetal:placental weight ratio	-	6.5 (±1.6)	-	9.3 (±1.3)	-	< 0.001[a]

a = significantly different p ≤0.05
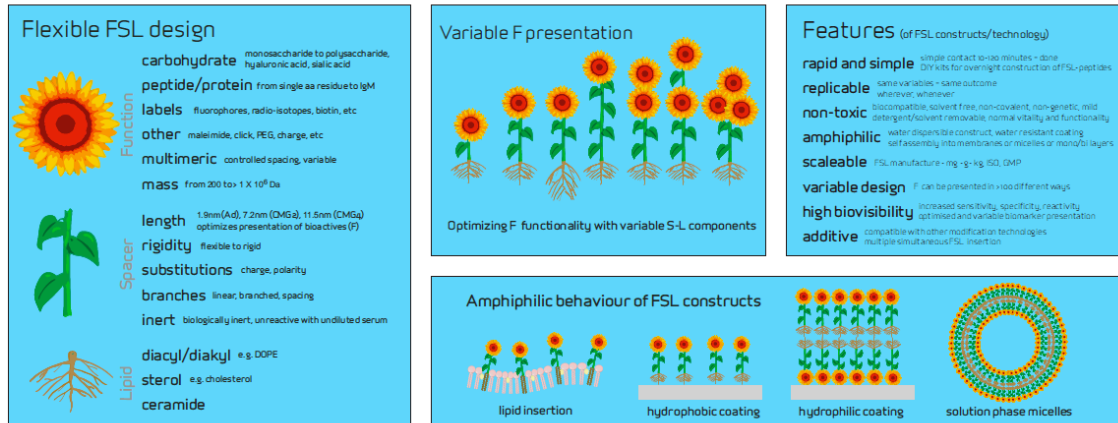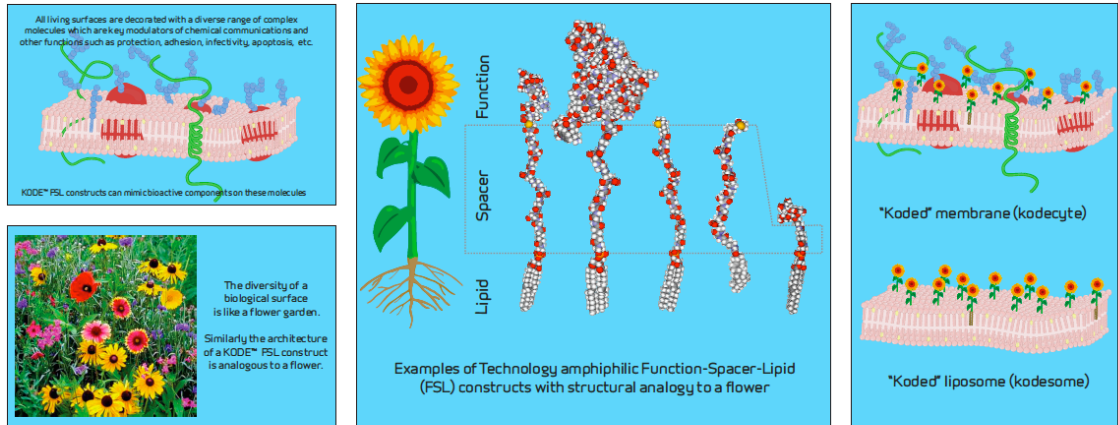
Figure A1 Poster explaining KODE™ FSL-technology. Retrieved with permission from http://kodebiotech.com

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